2016

Antiproliferative Activity of Sword Bean Lectin and Carbonate Apatite Against Ehrlich Ascites Carcinoma Cell

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ANTIPROLIFERATIVE ACTIVITY OF SWORD BEAN LECTIN AND CARBONATE APATITE AGAINST EHRLICH ASCITES CARCINOMA CELL

Ph D Thesis

A Dissertation

Submitted to the University of Rajshahi in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy in Applied Chemistry & Chemical Engineering.

Submitted by

Examination Roll No. 10703

Registration No. 40381

Session 2010-2011

Examination -2016

PHARMACEUTICAL RESEARCH LABORATORY

DEPARTMENT OF APPLIED CHEMISTRY & CHEMICAL ENGINEERING

UNIVERSITY OF RAJSHAHI

JUNE, 2016

CERTIFICATE

This is to certify that the thesis entitled **"Antiproliferative Activity of Sword Bean Lectin and Carbonate Apatite Against Ehrlich Ascites Carcinoma Cell"** has been prepared by Momtaz Fatima Waheed under my supervision for submission to the Department of Applied Chemistry & Chemical Engineering, University of Rajshahi, for the Degree of Doctor of Philosophy in Applied Chemistry & Chemical Engineering. It is also certified that the materials included in this thesis are original research work and have not been previously submitted for the award of any other degree.

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DECLARATION

I do hereby declare that the whole work submitted as a thesis entitled **"Antiproliferative Activity of Sword Bean Lectin and Carbonate Apatite Against Ehrlich Ascites Carcinoma Cell"** to the Department of Applied Chemistry & Chemical Engineering, University of Rajshahi, Bangladesh for the Degree of Doctor of Philosophy in Applied Chemistry & Chemical Engineering, are the original research work and have not been previously submitted elsewhere for the award of any other degree.

June, 2016

Momtaz Fatima Waheed
Ph.D. Research Fellow

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Abstract

A glucose specific lectin has been purified from the seeds of *Canavalia gladiata* (sword bean) in a single step by affinity chromatographic technique. For protein extraction, delipidated sword bean meal was homogenized with 100 mM sodium acetate-acetic acid buffer pH 5.5 for 5 hours at 4-6^{o}C. The clear supernatant was separated by centrifugation at 11,000 rpm for 10 minutes at 4-6^{o}C and designated as crude extract. The crude extract was subjected to 90% ammonium sulfate precipitation for overnight at 4-6^{o}C. After centrifuge at 11,000 rpm for 10 minutes 4-6^{o}C, the precipitate was dissolved in 100mM sodium acetate-acetic acid buffer pH 5.5 containing 1mM $MnCl_2$, 1mM $CaCl_2$ and 100mM NaCl. The protein solution was dialyzed against the same buffer to remove remaining ammonium sulfate. After dialysis, the sample was centrifuged and the clear supernatant was injected to the affinity column packed with Sephadex G-200 gel and equilibrated with binding buffer (100mM sodium acetate-acetic acid buffer pH 5.5 containing 1mM $MnCl_2$, 1mM $CaCl_2$ and 100mM NaCl). The column was washed to remove unbound materials. Gel-bound protein was eluted with 100mM sodium acetate-acetic acid buffer pH 5.5 containing 0.4M glucose. Finally, in order to remove salts and glucose from the eluted protein solution, it was dialyzed against deionized distilled water.

The purity of the protein was checked by 12.5% SDS-PAGE method. The SDS-PAGE gel shows five bands in the lane of crude extract and single band in the lane of purified protein, which indicates the affinity matrix system was able to purify only one kind of protein in a single step. The molecular weight of the affinity purified sword bean lectin (SBL) was determined and it was found to be 28 KDa.

The purified sword bean lectin powerfully agglutinate human blood types A, B, O erythrocytes almost equally. The order of hemagglutination activity with chicken and mice erythrocytes was mice>> chicken> human blood types A, B and O groups but not with the bovine and goat erythrocytes. This SBL was stable upto 60°C and showed maximum hemagglutinating activity at pH 7.5. The sugar inhibition assay exhibited its sugar specificity highly against D(+) glucose at Minimal Inhibition Concentration (MIC) of 25 mM and maltose, D(-) mannose, fructose at MIC of 3.125 mM. But lectins were not inhibited by the presence of D(+) galactose, L(+) arabinose.

Antiproliferation activity of purified SBL was tested against Ehrlich Ascites Carcinoma (EAC) cell in the swiss albino mice at a dose of 4 mg/kg/day and it worked as an active inhibitor. The result was compared with a known effective anticancer drug, bleomycin at the dose of 0.3 mg/kg/day (i.p.). The cell growth inhibition was found to be 77% and 90% for

SBL and bleomycin respectively. RBC level of EAC cell bearing mice increased to 8.6 which is very close to normal value of 9.4. The hemoglobin level also increased to 10.0 which is near to normal value of 11.4 gm/dl.

Carrier of various drugs and bio-active molecules such as peptides, proteins and DNAs etc. through the cell membrane into cells has attracted increasing attention because of its importance in medicine and drug delivery. Therefore, SBL combining with inorganic carbonate apatite as drug carrier in different concentration was also used to treat EAC cell bearing mice to get a better result, but the result was not satisfactory. The combined effect of SBL and carbonate apatite decreased EAC cell growth inhibition from 77% to 47% and 45% to 9% when 5mM carbonate apatite (4 ml/kg/day) was used with SBL at the dose of 4mg/kg/day and 2.5 mg/kg/day respectively whereas 5mM carbonate apatite at the same dose itself gave a very good inhibition of 70%. The RBC and hemoglobin level increased after treating with carbonate apatite to 8.0 and 10.9 which is nearer to normal level of 9.4 and 11.4 respectively. Fluorescence study of SBL and SBL combined with carbonate apatite showed that surface induced denaturation of SBL was occurred when it was administered in combination with carbonate apatite. As a result it prohibit the individual antiproliferation effect of sword bean lectin and carbonate apatite.

1.1 Introduction

1.1.1 Lectins

Lectins are sugar-binding proteins which are highly specific for their sugar moieties. They typically play a role in biological recognition phenomena involving cells and proteins. For example, some viruses use lectins to attach themselves to the cells of the host organism during infection. The name "lectin" is derived from the Latin word *legere*, meaning, among other things, "to select".

Although they were first discovered more than 100 years ago in plants, they are now known to be present throughout nature. It is generally believed that the earliest description of such a hemagglutinin was by Peter Hermann Stillmark in his doctoral thesis presented in 1888 to the University of Dorpat, (one of the oldest universities in czarist Russia). This hemagglutinin, which was also highly toxic, was isolated by Stillmark from seeds of the castor tree (*Ricinus communis*) and was named ricin. The first lectin to be purified on a large scale and available on a commercial basis was concanavalin A, which is now the most used lectin for characterization and purification of sugar-containing molecules and cellular structures.

1.1.2 Classification and properties of lectins

In the modern classification, there are three main types of lectins:

1. C-type
2. S-type and
3. Legume.

1. **C- type lectins**: C-type plant lectins are characterized by a calcium dependent carbohydrate recognition domain. There are two main types of C-type lectins.

a. Mannose binding protein and
b. Selectins.

2. **S-type lectins**: S-type lectins (S for soluble) or galectins are small, soluble proteins with calcium independent affinity for lactosamine and β- galactoside. There are three main S-type lectins.

a. S-lac
b. Galactose binding and
c. Galectin.

3. **Legume lectins**: They have both calcium and manganese binding sites.

But lectins are usually classified on the basis of source. They are of various types: plant lectin, vertebrate lectin, invertebrate lectin, lectin of slime mold, lectin of protozoa, viral lectin and bacterial lectin according to their respective sources. Regardless of source, all of the effects of lectins are believed to be a manifestation of the ability of the lectins to specific kinds of sugar, thereby agglutinate red blood cells of various animals and possess many other characteristic properties such as exert wide range of biological effects on the cell. Some of the properties of each group of lectin are given below:

Animal lectins: According to sources, there are three types of animal lectins. Those are (a) Vertebrate lectins (b) Invertebrate lectins and (c) Bacterial surface lectins.

Vertebrate lectins: Vertebrate lectins are divided into two classes, these are, (i) Integral membrane lectins that require detergents for their extraction; and (ii) soluble lectins (Barondes, S. H., 1981; Barondes, S. H. 1984). The first group comprises of lectins that differ in their sugar specificities (mannose, L-fructose, mannose-6-phosphate, N-acetylgalactosamine) and physicochemical properties (Ashwell and Harford, 1982). Among the best characterized lectins, the receptor for the mannose-6-phosphate is present in a variety of cells (Hasilik and Vonfigura., 1986) of the soluble vertebrate lectins, the first to be purified was the β-galactoside specific lectins from the electric organ of the eel *Electric electricus*. The soluble β-galactoside specific vertebrate lectins are of a similar molecular size, consisting each of two subunits of molecular weight 13,500 to 16,500 (Barondes, S. H. 1984) and require a reducing agent to maintain their carbohydrate binding activity.

Invertebrate lectins: These lectins are found in approximately 30 phyla and in the various classes and sub-classes of invertebrates (Cohen,1984; Gold and Balding,1975), mainly in the hemolymph and sexual organs, *e.g.* albumin glands and eggs. They are also present in the membranes of hemocytes, cells that function as primitive and rather unspecific immunological protectors (Cheng *et al.,* 1984; Vasta *et al.,* 1984). It has become clear that lectins are present in snails (Sharon, N., 1986). Recently Vivian Teichberg at the Weizmann Institute of Science in Israel discovered a lectin in the electric eel (Teichberg *et al.,* 1975). These substances have also been referred to as Phytohemagglutinins, although the term phytolectin has been proposed in

order to distinguish those lectins which are found in plants from those which are of animal or microbial origin.

Bacterial surface lectins: Many intact bacteria possesses the ability to bind and agglutinate erythrocytes and other types of cells (Mirelman, 1986). These activities are frequently inhibited by sugars, suggesting the presence of lectins on the bacterial surfaces.

Plant lectins: Plant lectins are the first and still the largest and best characterized group. It becomes apart that many of the lectin could be grouped into families with sequence homologies and common structural properties. The largest and the best-characterized family is that of the *Leguminoseae* lectins. Small families, also of plant lectins, are those from Nymphaeaceae (*e.g.* white water lily tuber) lectin.

The main sources of lectins are mature seeds and tubers. Similar to the major reserve protein of the seeds, the bulk of the lectin is located in the cotyledons in organelles known as protein bodies. Small amount of lectin are present in other tissues such as leaves, bark, and roots (Etzler, M.E, 1986). Most plant tissues contain one lectin, but in some cases two (or more) lectins that differ in their sugar specificities and other properties. They are classified into a small number of specificity groups (Mannose, L-fructose, N-acetylglucosamine, N-acetylgalactosamine, galactose and N-acetylneuramic acid) according to the monosaccharide that is the most effective inhibitor of the agglutination of erythrocytes.

1.1.3 Biological properties of lectins

Since virtually all cells come in a sugar coating, it is not surprising that lectins binds readily to cells. Such binding may result in a variety of biological effects.

1.1.3.1 Agglutination

Agglutination is the most easily detectable manifestation of the interaction of a lectin with cells, and to this very day is used to reveal the presence of a lectin in a biological source. The ability to agglutinate cells distinguishes lectins from other sugar-binding macromolecules, such as glycosidases and glycosyltransferases, and is therefore included in the definition of lectins according to Goldstein *et al.,* (1980).

For agglutination to occur the bound lectin must form multiple cross bridges between apposing cells. There is, however, no simple relation between the amount of lectin bound and

agglutination. Cases are even known where considerable amounts of a lectin are bound to cells without causing agglutination. This is because agglutination is affected by many factors such as the molecular properties of the lectin (*e.g.* number of saccharide binding sites, molecular size), cell-surface properties (for example, number and accessibility of receptor sites, membrane fluidity), and metabolic state of the cells (Nicolson, G.L. 1976). In addition, agglutination is affected by external conditions of assay such as temperature, cell concentration, mixing and so on. Agglutination is inhibited by appropriate sugar.

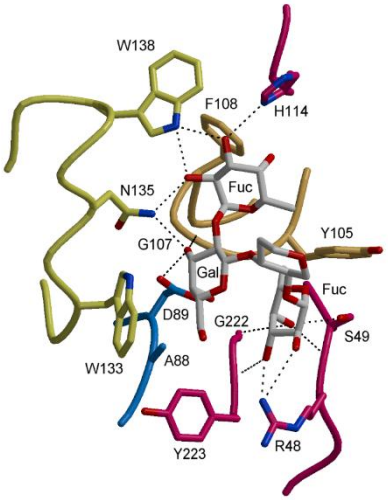


Figure 1-1: An oligosaccharide (shown in grey) bound in the binding site of a plant lectin. Only a part of the oligosaccharide (central, in grey) is shown for clarity.

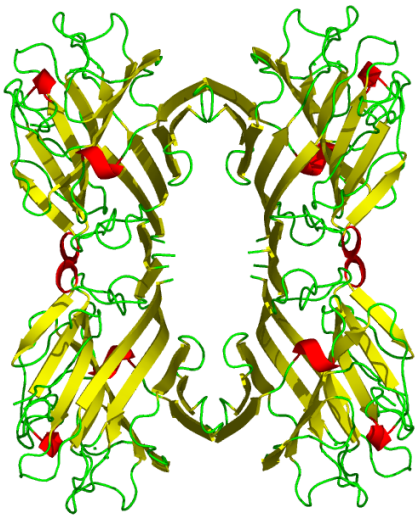


Figure 1-2: Leucoagglutinin, a toxic phytohemagglutinin found in raw *Vicia faba*.

1.1.4 Role and applications of lectins

1.1.4.1 Biological role and applications of lectins

Lectins are currently attracting much interest, primarily because they serve as invaluable tools in diverse areas of biomedical research. Because of their unique carbohydrate binding properties, lectins are useful for the separation and characterization of glycoproteins and glycopeptides, in studies of glycolipids, following changes that occur on cell surfaces during physiological and pathological processes, for cancer treatment due to its anticancer activity, for histochemical studies of cells and tissues, for tracing neuronal pathways, typing blood cells and bacteria, for fractionation of lymphocytes and of bone marrow cells for bone transplantation. They are also used to stimulate lymphocytes to assess the immune state of patients and for chromosome analysis in human cytogenetics, as well as for the production of cytokines. In addition, lectins are excellent models to examine the molecular basis of specific reactions that occur between proteins and other types of molecules, both of low or high molecular weight, such as the binding of antigens to antibodies, of substrates to enzymes, of drugs to proteins and of hormones and growth factors to cells. lectins can also be used in protein chemistry for column packing in affinity chromatography *e.g.* concanavalin A lectin and wheat germ agglutinin. lectins also have biomedical importance as they can be used for ulcer treatment.

Dietary lectins can cause diseases if the individual is lectin sensitive (because of genetic and immunogenic abnormalities and also by viral or bacterial infections). There are some properties of dietary lectins that are responsible for causing those diseases:

(a) lectins are hardy proteins that do not break down easily. They are resistant to stomach acid and digestive enzymes.

(b) lectins may bind to the gut wall and damage the gut lining and may pass through the gut into general circulation.

(c) lectins can cause alterations in gut function that may be related to colitis, Crohn's Diseases, Celiac-Sprue, IBS (Irritable bowel syndrome) and gut permeability.

(d) lectin damage to the gut wall may allow other non-lectin proteins to cross undigested into general circulation and cause allergic reactions, including anaphylaxis.

(e) Having gained access to general circulation, lectins may bind to surface cell membranes of organs and glands including the thyroid, pancreas, kidney and adrenals, in susceptible animals and humans.

(f) This binding may begin antigen-antibody reactions leading to autoimmune disorders and so-called degenerative diseases.

(g) Dairy lectins may be implicated in juvenile onset type 1 diabetes. Wheat lectins may be implicated in juvenile nephropathy.

1.1.4.2 Role of lectins in nature

The roles of lectins in nature are given below:

i) Plant source

(a) Attachment of nitrogen-fixing bacteria to legumes.
(b) Protection against phytopathogens.

i) Animal source

(a) Endocytosis and intra-cellular translocation of glycoproteins.
(b) Regulation of cell migration and adhesion.
(c) Recognition of determinants in nonimmune phagocytosis.
(d) Binding of bacteria to epithelial cells.

i) Microorganism source

(a) Attachment of bacteria and parasites (*e.g*. amoeba and plasmodium) to host cells.
(b) Recognition of determinants in non-immune phagocytosis.
(c) Recognition of determinants in cell adhesion of slime molds.

1.1.5 Use in science, medicine and technology

1.1.5.1 Use in medicine and medical research

Purified lectins are important in a clinical setting because they are used for blood typing (N. Sharon). Some of the glycolipids and glycoproteins on an individual's red blood cells can be identified by lectins.

a) A lectin from *Dolichos biflorus* is used to identify cells that belong to the A1 blood group.
b) A lectin from *Ulex europaeus* is used to identify the H blood group antigen.
c) A lectin from *Vicia graminea* is used to identify the N blood group antigen.
d) A lectin from *Coconut milk* is used to identify Theros antigen.

e) A lectin from *Dorex* is used to identify R antigen.

In neuroscience, the anterograde labeling method is used to trace the path of efferent axons with PHA-L, a lectin from the kidney bean (Carlson, Neil R. 2007).

A lectin (BanLec) from bananas inhibits HIV-1 *in vitro* (www.jbc.org/content/285/12/8646).

1.1.5.2 Use in studying carbohydrate recognition by proteins

Lectins from legume plants, such as PHA or concanavalin A, have been widely used as model systems to understand the molecular basis of how proteins recognize carbohydrates, because they are relatively easy to obtain and have a wide variety of sugar specificities. The many crystal structures of legume lectins have led to a detailed insight of the atomic interactions between carbohydrates and proteins.

1.1.5.3 Use as a biochemical tool

Concanavalin A and other commercially available lectins have been widely used in affinity chromatography for purifying glycoproteins (www.gelifesciences.com). In general, proteins may be characterized with respect to glycoforms and carbohydrate structure by means of affinity chromatography, blotting, affinity electrophoresis and affinity immunoelectrophoreis with lectins as well as in microarrays as in evanescent-field fluorescence-assisted lectin microarray (Glyco Station, Lec Chip, Glycan profiling technology (http://www.gpbio .jp/english/tech.html).

1.1.5.4 Use in biochemical welfare

One example of the powerful biological attributes of lectins is the biochemical warfare agent ricin. The protein ricin is isolated from seeds of the castor oil plant and comprises two protein domains. Abrin from the jequirity pea is similar:

- One domain is a lectin that binds cell surface galactosyl residues and enables the protein to enter cells.
- The second domain is an N-glycosidase that cleaves nucleobases from ribosomal RNA, resulting in inhibition of protein synthesis and cell death.

1.1.6 The toxic effect of lectins and their role as anticancer agents

Both oncogenic (Abu *et al.,* 1963) and non-oncogenic, virally transformed cells possess increased susceptibility to agglutination by lectin, that exhibit as a toxic substance even in very small amount to higher animals. Abrin and ricin possess such kinds of characteristics. They inhibit protein synthesis (Lin *et a*l., 1970) as well as DNA and also RNA in the cell of culture.

A tumor inhibiting effect of ricin was reported. Lin *et al.,* (1969) demonstrated that abrin and ricin were able to prevent the development of ascites tumor even when administered five days after inoculation of the tumor cells. The toxins have also been tried in the treatment of human cancer, particularly uterine cancer.

1.1.7 Introduction to the plants used in this study

The legume lectins are a family of sugar binding proteins or lectins found in the seeds and in smaller amounts, in the roots, stems, leaves and bark of plants belonging to the Fabaceae family (Loris *et al.*, 1998). Maltose binding protein includes a class of enzyme extensively distributed in a wide range of plant species (*e.g. Canavalia enrisformis, C. gladiata, C. cathartica,* etc.). The commercially available maltose binding protein comes from jack bean (*Canavalia enrisformis*), which does not grow in Bangladesh. Based on a previous screening experiment in our laboratory, it has been shown that sword bean contain considerable amount of lectin. So in our research laboratory we have used sword bean seeds (*C. gladiata*).

1.1.7.1 Sword bean

The common name of the *Canavalia gladiata* is the "Sword Bean", due to the seed pod's resemblance to the blade of a sword. The sword bean is also known under other names, including Sword bean, Scimitar bean, Jamaican horse bean, Kacang Parang (Malay), Khadsampal, Badi sem (Hindi), Tebi (Manipuri), Segapputampattai (Tamil), Valpayar, Valaringha (Malayalam), Tamma (Telugu), Tumbekonti, Sembi (Kannada), Makhan shim, Kutra shim (কাটরা সিম) (Bengali), Mahasimbi, Asisimbi (Sanskrit). Botanically, it is known as *C. gliadata* and originates from tropical Asia and Africa. Sword been fruit, approximately 20–40 cm long 3.5–5 cm width; seeds are red or red-brown, 2–3.5 cm long.

Figure 1-3: Photograph of sword bean seeds

Figure 1-4: Photograph of sword bean.

Scientific classification	
Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Fabales
Family:	Fabaceae
Genus:	*Canavalia*
Species:	*C. gladiata*
Binomial name	
Canavalia gladiata (Jacq.) DC.	

1.1.8 Aim of the present investigation

Legumes, being a cheap source of high protein content are a major food source for both human and animals in the world, due to their nutritional benefits such as high in fiber and low in fat (Muzquiz *et al*., 1999; Sathe S.K, 2002). Legume lectins are large family of homologous carbohydrate binding proteins. Lectin can be defined as glycoproteins of nonimmune origins that are capable in the recognition and reversible binding to carbohydrate moieties without the alteration of the covalent structure of the recognized glycosyl ligands (Vasoconcelos *et al.*, 2004; Macedo *et al.,* 2003). Because of their distribution in all tissues of plants, it has been suggested that lectins provide an important role in plants as a defense mechanism and in the recognition of nitrogen fixing bacteria (Vasoconcelos *et al.*, 2004; Macedo *et al.,* 2003; Chrispeels and Raikhel, 1991; Sharon and Lis, 2004). Lectins serve many different biological functions, from the regulation of cell adhesion to glycoprotein synthesis and the control of protein levels in the blood. They also display anti-tumor (Pusztai *et al* 1998), immunomodulatory (Abdullahev and Meija., 1997), anti-fungal (Rubinstein *et al.*, 2004), anti-human immunodeficiency virus (HIV) (Herre *et al*., 2004), and anti-insect (Barrientos and Gronenborn, 2005) activities. It has been long known that these proteins are capable of agglutinating red blood cells in mammals, including all human blood types (Felsted *et al.,* 1981). Thus, the purified lectins are important in a clinical setting because they are used for blood typing. Some of the glycolipids and glycoproteins in an individual's red blood cells can be identified by lectin. Other functions are its ability to bind to the surface of the intestinal walls. They have also been associated with the appearance of lesions, disruptions and abnormal growth of the microvilli, as well as the malabsorption of nutrients across the membranes (Chrispeels *et al.,* 1997). Recent studies have shown that these compounds possess anti-HIV activity. In analytical biochemistry, lectins are used as a tool for affinity purification of glycoproteins *e.g.* glucose oxidase, peroxidase etc. A lectin was isolated from Erythrina speciosa (Konozy, E.H. *et al.* 2003) by affinity chromatography and was characterized as a D-galactose binding lectin which showed specific variations from other members in its carbohydrate binding site and biological activity which is due to the importance of Ca^{2+} and Mn^{2+} ions for its activity. Among the lectins, Con A was the first lectin to be isolated in the pure form (Sumner J.B,1919) . It occurs in high concentration in the jack bean (*Canavalia ensiformis*). Purified Con A can be used for glycoprotein enrichment by a nanoscale, chelating, Con A monolithic capillary prepare by using GMA-EDMA (glycidyl methacrylate-co-ethylene dimethacrylate) as polymeric support. Literature shows that jack bean *(Canavalia ensiformis)* is a rich source of Con A. But this plant does not grow well in Bangladesh. Based on previous screening experiment in our laboratory on Bangladeshi plant species, it has been found that

sword been seeds contain considerable amount of lectin. The aim of the present investigation is to extend our works towards

1. **Isolation and purification of lectin from sword bean.**
2. **Purity and molecular weight determination of the lectin.**

1.1.9 Literature review

The concept of resolving complex macromolecules by means of biospecific interactions with immobilized substrates has its antecedents reaching back to the beginning of the 20th century. The German pharmacologist Emil Starkenstein (1884-1942) in a paper published in 1910 (Starkenstein, E.V,1910) on the influence of chloride on the enzymatic activity of liver α-amylase was generally considered to be responsible for the first experimental demonstration of the biospecific adsorption of an enzyme onto a solid substrate, in this case, starch. Not long after, Willstätter *et al*. (Willstatter *et al.,* 1923) appreciably enriched lipase by selective adsorption onto powdered stearic acid. It was not until 1951, however, that Campbell and co-workers (Campbell *et al.,* 1951) first used the affinity principle to isolate rabbit anti-bovine serum albumin antibodies on a specific immunoadsorbent column comprising bovine serum albumin coupled to diazotized *p*-aminobenzyl-cellulose. This technique, now called immunoaffinity chromatography, became established before the development of small-ligand selective chromatography, where Lerman, L.S, 1953 isolated mushroom tyrosinase on various *p*-azophenol-substituted cellulose columns, and Arsenis and McCormick, 1964 and Arsenis *et al.,* 1966 purified liver flavokinase and several other FMN-dependent enzymes on flavin-substituted celluloses. Insoluble polymeric materials, especially the derivatives of cellulose, also found used in the purification of nucleotides (Sander *et al.,* 1966), complementary strands of nucleic acids (Bautz and Hall 1962) and certain species of transfer RNA (Erhan *et al.,* 1965).

The general notion of exploiting strong reversible associations with highly specific substrates or inhibitors to effect enzyme purification was evident in the literature in the mid-1960s (McCormick, 1965), although the immense power of biospecificity as a purification tool was not generally appreciated until 1968 when the term 'affinity chromatography' was coined (Cuatrecasas *et al.,* 1968).

A further key development introduced in the early 1970s was that of 'group-specific' (Lowe and Dean, 1971) or 'general ligand' (Mosbach *et al.,* 1972) adsorbents. An important advantage of ligands with a broad bioaffinity spectrum, such as the coenzymes, lectins, nucleic acids, metal chelates. Protein A, gelatine and heparin, is that it was not obligatory to devise a new

organic synthetic strategy for every projected biospecific purification. However, a possible disadvantage of the group-specific approach is that the broad specific of the adsorption stage required a compensatory specific elution step to restore the overall biospecificity of the chromatographic system. Nevertheless, of the thousands of enzymes that have been assigned a specific Enzyme Commission number, approximately one-third involve one of the four adenine coenzymes (NAD^+, $NADP^+$, CoA and ATP), and not surprisingly, these classes of enzymes were the first to be targeted by this approach (Mosbach *et al.*, 1971) and subsequently extensively exploited in the purification of oxido-reductases by affinity chromatography and in enzyme technology (Harvey *et al.,* 1974; Lowe *et al.,* 1974; Lowe *et al.,* 1980).

Until this point in time, most of the studies had generated rules-of-thumb on how to apply the technique of affinity chromatography to selected purifications. However, it became apparent on even a rudimentary examination of the theoretical basis of the technique (Lowe *et al.*, 1974) that the implicit assumption that the observed chromatographic adsorption of the target protein to the immobilized ligand was due exclusively to biospecific enzyme-ligand interactions was misguided. The large discrepancies observed between what was anticipated on the basis of the biological affinity for the immobilized ligand and what was observed experimentally to be the case were found to be due to the largely unsuspected interference by non-biospecific adsorption, which in many cases, completely eclipsed the biospecific adsorption (O'Carra *et al.*, 1973). O' Carra and co-workers (O'Carra *et al.*, 1974) demonstrated that spacer arms do not always act simply as passive links between biospecific ligands and the polymer matrix and described methods for the control of interfering nonspecific adsorption effects and for the optimization of affinity chromatography performance by a logical and systematic appraisal of reinforcement effects and, where applicable, kinetic and mechanistic factors. Whilst the necessity for spacer arms interposed between the ligand and matrix was recognized very early in order to alleviated steric interference (Cuatrecasas *et al.,* 1968; Lowe *et al.,* 1973; Hipwell *et al.,* 1974), it was not until later that it was realized that the aliphatic hydrocarbons commonly employed as spacers could act as hydrophobic ligands in their own right. In a study with pre-assembled AMP ligands containing spacer arms of varying degrees of hydrophilicity and hydrophobicity, it was found that enzymes bound preferentially to ligands tethered via hydrophobic spacer arms and that the notion of constructing adsorbents comprising a ligand attached to a matrix via a hydrophilic arm in order to ameliorate nonspecific hydrophobic interactions may not be a viable proposition (Lowe *et al.,* 1977). Alternative strategies of combating these undesirable effects, such as inclusion of low concentrations of water-miscible organic solvents in the buffers (e.g. ethylene glycol, glycerol or dioxane), were adopted as they resulted in dramatically improved recoveries of the released enzyme (lowe and Mobach, 1975).

One of the most widely used combinatorial technologies is based on biological vehicles as platforms for the presentation of random linear or constrained peptides, gene fragments, cDNA and antibodies. The non-lytic filamentous bacteriophage, M13, and the closely related phages, fd and fl, are the most commonly exploited vectors with random peptides displayed on the surface of the phage by fusion of the desired DNA sequence with the genes encoding coat proteins (Burritt *et al.,* 1996; Katz, 1997). Combinatorial libraries containing up to 10^9 peptides can be generated and selected for the desired activity by 'bio panning' of the phage pool on a solid-phase immobilized target receptor. Bound phage particles are eluted, amplified by propagation in *Escherichia coli* and the process repeated several times to enrich iteratively for the peptide with the desired binding properties, and whose sequence is determined from the coding region of the viral DNA. Phage display libraries have been successfully applied to epitope mapping, vaccine development, the identification of protein kinase substrates, bioactive peptides and peptide mimics of non-peptide ligands and are eminently suitable as source of affinity ligands for chromatography or analysis (Goldman *et al.,* 2000). However, a limitation of the phage display approach is that peptides may only function when the peptide is an integral part of the phage-coat protein and not when isolated in free solution (Jense-Jarolim *et al.,* 1999). These limitations can be circum-vented to some extent by using conformational constrained peptides (Kim *et al.,* 2000), although issues relating to retention of their function on optimization, scale-up and use on various solid-phase matrices still remain (Lam *et al.,* 1991). An alternative approach based on ribosome display for the evolution of very large protein libraries differs from other selection techniques to that the entire procedure is conducted in vitro and is particularly appropriate for the screening and selection of folded proteins (Hanes *et al.,* 2000). Other scaffolds exploiting domains from proteins such as fibronectin ('monobodies'), V domains ('minibodies') or α-helical bacterial receptor domains ('affibodies') have been shown to yield specific binders, with usually mM affinities, from libraries of up to 10^7 clones (Nygren *et al.,* 1997).

A significant development in affinity techniques for proteomics is the use of fusion tags or proteins for expression and purification (Derewenda, 2004; Waugh, 2005; Bhikhabhai *et al.,* 2005). A large choice of systems is available for expression in bacterial hosts, with a further selection amenable for eukaryotic cells. Amongst the most popular fusion partners for molecular, structural and bioprocessing applications are the polyArg (Sassenfeld and Brewer, 1984), hexaHis-tag (Smith *et al.,* 1988), glutathione-S-transferase (Smith *et al.,* 1988) and maltose-binding protein (Di Guan *et al.,* 1988). Other less commonly employed expression tags include thioredoxin (La vallie *et al.,* 1993), the Z-domain from Protein A (Nilsson *et al.,* 1987), NusA (Whetstone *et al.,* 2004), GBI domain from Protein G (Davis *et al.,* 1999) and others

(Balbas, P., 2001). A recent comparison of the efficiency of eight elutable affinity tags for the purification of proteins from *E. coli*, yeast, *Drosophila* and HeLa extracts shows that none of these tags is universally superior for a particular system because proteins do not naturally lend themselves to high throughput analysis and they display diverse and individualistic physicochemical properties (Huth *et al.,* 1997). It was found that the His-tag provided good yields of tagged protein form inexpensive, high capacity resins but with only moderate purity form *E. coli* extracts and poor purification form the other extracts. Cellulose-binding protein provided good purification form HeLa extracts. Consequently, affinity tags are invaluable tools for structural and functional proteomics as well as being used extensively in the expression and purification of proteins. Affinity tags can have a positive impact on the yield, solubility and folding of their complementary fusion partners. Combinatorial tagging might be the solution to choosing the most appropriate partner in high throughput scenarios (Lchty *et al.,* 2005).

Relatively few lectins have been purified from plants. Only recently have much attention been given to the isolation, determination of content and properties of legume root lectins (Horejsi *et al., 1978;* Gatehouse and Boulter, 1980; Quinn and Etzler, 1987). As reported earlier, a lectins known as trifoliin has been isolated from the root of white clover (*Trifolium repens*) (Lis and Sharon, 1986; Sharon and Lis, 1989). This root lectin was purified and was found to be similar in immunological and electrophoretic properties to white clover seed lectin (Dazzo *et al.,* 1978). Also, roots of soybean (*Glycine max*) have hemeagglutinating activity and contain a protein which reacted with antibodies directed against soybean seed lectin. Thus, it was then assumed that seed lectin was the same as root lectin (Gatehouse *et al* 1980). However, more recent studies have revealed that this was not the case (Gatehouse *et al* 1980; Quinn and Etzler, 1987).

The difference between root lectins and seed lectins was demonstrated through tests on the specificity of root lectins binding to *Rhizobia* conducted by Bohlhool and Schmidt (1974), Chen and Phillips (1976), Dazzo and Brill (1977), Law and Strijdom (1977) and Bhuvanesurai and Bauer (1978). As reviewed by Gatehouse and Boulter (1980), root lectin from *Pisum sativum* (pea) gave a different banding pattern in isoelectric focusing as compared to its seed lectin (Gatehouse *et al* 1980). In addition, the two lectins gave different carbohydrate specificities when assayed using sugar inhibition (Gatehouse *et al* 1980). Quinn and Etzler (1987) pointed out differences in amino acid composition between the root and seed lectins from *Dolichos biflorus* (horsegram). They also discovered differences in the banding patterns of the two lectins in isoelectric focusing.

The search for the toxic principle in castor beans (*Ricinus communis*, Euphorbiaceae) prompted one of the most prominent pharmacologists of his time, Rudolf Kobert (1854–1918),

to ask his medical student Hermann Stillmark (1860–1923) to study this plant. In his thesis of 1888 at the University of Dorpat/Tartu, Stillmark described that extracts from castor beans and four other Euphorbiaceae plants are able to agglutinate blood cells from different animals, i.e. rabbits, horses, dogs and cats. He assumed toxicity and agglutinating capability to originate from the same substance, ricin, which he regarded to be an enzyme (in those days "ferment") (Stillmark *et al.,* 1888). As we now know, castor beans contain a tetrameric protein called *Ricinus communis* agglutinin that is able to agglutinate cells but is hardly toxic. A second, dimeric protein, now called ricin or *Ricinus* toxin, is closely related to the agglutinin, and its enzymatic subunit acts as a highly specific RNA N-glycosidase on 28S rRNA but is only a weak agglutinin. This observation on agglutinating activity resembles that made by S. Weir Mitchell already in 1860, i.e. the activity of rattlesnake (*Crotalus durissus*) venom on pigeon's blood (Mitchell *et al.,* 1860).

Soon after Stillmark's discovery, ricin and the related toxin from jequirity beans (*Abrus precatorius,* Leguminosae) played a fundamental role as model antigens in the pioneering studies of Paul Ehrlich (1891). When passing through ricin's history and of note in the era of heightened awareness of biohazard, its extremely potent toxicity even attracted the attention of the Bulgarian secret service. In 1978, they turned it into a deadly weapon in the umbrella homicide of the exile-Bulgarian Georgi Markov who worked for BBC London, prompting infamous headlines for a lectin (Knight, 1979).

Looking back to the beginning of lectin research, progress in the field was hampered in the first decades by the crudeness of the fractionation techniques. The first agglutinin to be isolated was concanavalin A from Jack bean (*Canavalia ensiformis*) seeds. The prominent American biochemist James B. Sumner, well-known for demonstrating that an enzyme (urease) is nothing but a protein (which earned him the Nobel award in 1946), succeeded in purifying also the agglutinating principle from these seeds by crystallization and called it concanavalin A (Con A) (Sumner *et al.,* 1919). He discovered that con A is able to interact with red blood cells as well as with starch, glycogen and mucins, and that this interaction can be prevented by low-molecular weight carbohydrates such as sucrose (Sumner *et al.,* 1936). This result was the first clear experimental indication that an agglutinin binds carbohydrates and had significant implications for the study of cell membrane constituents. Systematic screening of plant extracts for agglutinating activities led to a breakthrough in haematology and paved the way for coining the term *lectin*.

In fact, Landsteiner's discoveries of human isoagglutinins in 1900 in Vienna and the species specificity of plant agglutinins as well as his comparison of haemagglutinating/haemolyzing

activities with natural antibodies led W. C. Boyd "to test seeds for blood group specificity" (Boyd, 1963). With extracts of the lima bean (*Phaseolus lunatus limensis*, Leguminosae) he found A-type specificity and proposed "the term 'lectin' (from the Latin *legere*, to choose or to pick out) for these and other antibody like substances" (Boyd and Shapleigh,1954). Drawing on the competitive inhibition of antibody-antigen reactions by compounds structurally related to the haptenic group described by Landsteiner and van der Scheer, 1931 and the binding of con A to sugar compounds documented by Sumner and Howell (1936), inhibition of haemagglutination mediated by eel (*Anguilla anguilla*) serum and extracts of *Lotus tetragonolobus* seeds by Fuc and of haemagglutination by other plant lectins by GalNAc provided first insights into the chemical nature of the blood group determinants (Morgan *et al.*, 1953). From this starting point, the chemical nature of the blood group substances as oligosaccharides could be delineated. For the reaction of red blood cells with blood-group-specific lectins, the terminal α- linked monosaccharides GalNAc (group A), Gal (group B) and Fuc (group 0(H)) are decisive. It goes without saying that these studies were further extended thereafter and were crucial for "unravelling the biochemical basis of blood group ABO and Lewis antigenic specificity" (Morgan *et al.*, 2000). With this focus on haemagglutination it is no surprise that the initial definition of the term *lectin* (Boyd *et al.*, 1954) placed special emphasis on just this aspect. As apparent from the example of the homologous lectin subunits of the *Ricinus* agglutinin/toxin, however, strict application of the criterion of haemagglutination would separate related proteins.

Thus, haemagglutination or precipitation of glycans which depend on at least bivalency are now considered as being only one example of a broad panel of assays able to detect carbohydrate binding (Gabius *et al.*, 1998). Moreover, lectins ought to be distinguished from other molecules able to clump erythrocytes together.

The synthesis of con A in *C. gladiata* starts 30 days after flowering and has different accumulation patterns during development. The synthesis and accumulation of con A increases gradually until seed maturation is nearly completed which is 80 days after flowering (Yamauchi *et al.*, 1986). Mialonier *et al.* (1973) separated the lectin in the cytoplasm of the cotyledon and the embryo and noticed its appearance during ripening and disappearance during germination. In a separate study on con A from *C. ensiformis*, though the protein appears in the seeds at day 24 after pod formation, there is a lag phase up to day 30 and a logarithmic increase to day 36 followed by a plateau up to the desiccation stage (Raychaudhuri *et al.*, 1988).

1.2 Materials and Methods

1.2.1 Preparation of solution and buffer

i) Preparation of 25mM phosphate buffer pH 7.5

Preparation of 25mM $NaH_2PO_4.2H_2O$ solution: 0.98 g of $NaH_2PO_4.2H_2O$ was dissolved in about 200 ml of deionized distilled water in a 250 ml volumetric flask. The final volume was made up to the mark by adding deionized distilled water.

Preparation of 25mM $Na_2HPO_4.2H_2O$ solution: 2.23 g of $Na_2HPO_4.2H_2O$ was dissolved in about 400 ml of deionized distilled water in a 500 ml volumetric flask. The final volume was made up to the mark by adding deionized distilled water. Before adjusting pH of the buffer, pH meter was calibrated from pH 7.0 to pH 10.0.

25mM $NaH_2PO_4.2H_2O$ solution was added drop wise into 25mM $Na_2HPO_4.2H_2O$ solution with proper stirring until pH of the solution reached to 7.5. Approximately 160 ml 25mM $NaH_2PO_4.2H_2O$ solution was required for 500 ml 25mM $Na_2HPO_4.2H_2O$ solution to adjust pH to 7.5. After adjusting the pH at 7.5, the solution was stored into a stoppered bottle at 10-15^oC.

ii) Preparation of 100mM sodium acetate-acetic acid buffer pH 5.5 containing 1mM $MnCl_2$, 1mM $CaCl_2$ and 100mM NaCl: 13.61 g of sodium acetate trihydrate ($NaC_2H_3O_2.3H_2O$) was dissolved in about 900 ml of deionized distilled water in a 1000 ml beaker. 0.20 g $MnCl_2$, 0.11 g $CaCl_2$ and 5.84 g NaCl were also dissolved in the solution.

Before adjusting pH of the buffer, the pH meter was calibrated from pH 4.0 to pH 7.0. pH of the solution was adjusted to 5.5 with acetic acid. The final volume was made upto the mark in a 1000ml volumetric flask adding deionized distilled water.

1.2.2 Column packing and preparation

1.2.2.1 Swelling of Sephadex G-200 gel:

The bed volume per g of dry Sephadex G-200 gel was 20-25ml. For swelling of Sephadex G-200 gel, deionized distilled water was filtered through 0.22 μm membrane to prevent microbial growth. Accurately weighed 1.5 g of dry Sephadex G-200 gel was added to sufficient volume (30% more than the expected bed volume) of filtered deionized distilled water in a beaker. It was kept at 90^oC temperature for 5 hours to accelerate the swelling process. After swelling was complete, the supernatant was decanted. Absolute alcohol (ethanol) was added to it to make the final concentration of ethanol 20% in the slurry.

1.2.2.2 Column packing

In this study, Sephadex G-200 gel was used as matrix in the chromatographic column to purify lectin. A slurry of Sephadex G-200 gel in 20% ethanol was degassed with the help of a vacuum pump. The HiScale column was set up vertically with the help of a stand. The lower end of the column was closed with the adapter and the degassed slurry was poured into the column through the upper opening in one continuous motion. To minimize the introduction of air bubbles, the slurry was poured down with glass rod held against the wall of the column. After settling the gel, the upper adapter of the column was mounted in proper way. Then the excess ethanol was allowed to pass through the tube connected with the lower adapter of the column to decrease the volume of the column. After that, binding buffer was passed through the column to remove ethanol from the column. At least three bed volume of binding buffer was passed through the column to remove ethanol completely from the column and equilibrate the column. Then the column was ready to load sample solution.

Figure 1-5: Photograph showing a HiScale empty column.

1.2.3 Sample Preparation

Samples for chromatographic purification should be clear and free from particulate matter. Simple steps to clarify a sample before beginning purification will avoid clogging the column, may reduce the need for stringent washing procedures and can extend the life of the chromatographic medium.

Sample extraction procedures and the selection of buffers, additives and detergents are determined largely by the source of the material, the stability of the target molecule, the chromatographic techniques that will be employed and the intended use of the product.

1.2.3.1 Collection and delipidation of seeds

The matured and dried sword beans were collected from cultivated field. The beans were peeled off to get red sword bean seeds. After removing the red seed coat, sword bean seeds were sun dried and pulverized in a pulverizer to produce fine powder materials. The powder materials were screened through 50 mesh screen. The fine powder obtained in this way is known as sword bean meal (briefly SBM). Sword bean meal thus obtained was defatted by slightly stirring with acetone in the ratio of SBM:acetone=1:2 for around 10 minutes. After that, the mixture was allowed to settle for 30 minutes at 4^{o}C. Then the acetone layer was removed from top of the settled SBM by siphoning. The defatted sword bean meal was air dried at room temperature to remove last traces of acetone and used as a starting material for lectin purification.

1.2.3.2 Extraction of protein

Defatted sword bean meal (20 gm) was subjected to protein extraction in 150 ml of 100mM Na-acetate-acetic acid buffer pH 5.5 with slightly stirring for 5 hours at 4-6^{o}C. The suspension was filtered through double layer of silk cloth. The filtrate was collected and centrifuged at 11,000 rpm for 10 minutes. The clear supernatant was taken and the precipitate was discarded.

1.2.3.3 Ammonium sulfate precipitation

The clear supernatant (100 ml) was subjected to 90% ammonium sulfate precipitation and kept overnight at 4-6^{o}C. On the next day, that was centrifuged at 11,000 rpm for 10 minutes to take the precipitate. The precipitate was dissolved in 100mM sodium acetate-acetate acid buffer pH

5.5 containing 1mM $MnCl_2$, 1mM $CaCl_2$ and 100mM NaCl salts. The solution was again centrifuged to get clear solution.

1.2.4 Dialysis

Dialysis is membrane transport process in which solute molecules are exchanged between two liquids. This process proceeds in response to the difference in chemical potential between two liquids or a liquid and a solid. The dialysis membrane is made of cellulose acetate, available in a wide range of dimensions and nominal molecular weight cut-offs (NMWC) allowing molecules below a certain molecular weight to freely equilibrate on both sides of the membrane. It is semi-permeable, meaning that molecules below a specified molecular weight can readily pass through the membrane, whereas larger molecules cannot. Dialysis membranes are available with a wide variety of molecular weight cut-offs; 10,000, 30,000 and 40,000 Dalton membranes are typically used for protein dialysis.

1.2.4.1 Activation of dialysis tubing

Dialysis tubing was activated by boiling in 250 ml solution containing 5 g $NaCO_3$ and 0.931 g EDTA disodium salt for 10 min followed by boiling in 250 ml distilled water. The tubing was then knotted carefully at one end, filled with distilled water and autoclaved at 100^{o}C for 10 minutes. Disposable gloves were worn when handling dialysis tubing.

1.2.4.2 Removing ammonia molecules by dialysis

The clear solution was dialyzed in 10kDa dialysis bag against 100mM sodium acetate-acetic acid buffer pH 5.5 containing 1mM $MnCl_2$, 1mM $CaCl_2$ and 100mM NaCl to remove excess ammonia. The presence or absence of ammonia in the dialysate was detected by Barthelot method (Rhine *et al.*, 1998). After dialysis the protein solution was centrifuged at 11,000 rpm for 10 minutes to get clear solution.

1.2.5 Determination of the protein content by Biuret method (Weichselbum *et al*., 1946)

Reagent preparation: The reagent was prepared by mixing the following solution.

Sodium hydroxide	200 mM
Potassium sodium tartrate	32 mM
Copper sulfate	12 mM
Potassium iodide	30 mM

Standard: Standard contains protein 80 gm/l and sodium azide 0.095%.

Procedure: 20 µl protein sample and 20 µl standard protein sample were added to 1.0 ml of total protein reagent in two different test tube. 20 µl of deionized distilled water was added to 1.0 ml of protein reagent in another test tube which was considered as blank. The mixtures were shaken to mix well, allowed to react for 10 minutes at room temperature. The absorbance of the sample and standard were measured against the reagent blank within 30 min at 546 nm using a UV-Vis Spectrophotometer. The amount of protein content in the protein sample was determined using the following equation.

$$C = 80 \times \frac{\Delta A_{sample}}{\Delta A_{standard}} [mg/ml]$$

1.2.6 Purification of lectin by Sephadex G 200 matrix

1.2.6.1 Preparation of buffers

i) Preparation of binding buffer (100mM sodium acetate-acetic acid buffer pH 5.5 containing 1mM $MnCl_2$, 1mM $CaCl_2$ and 100mM NaCl): Preparation of binding buffer was described before (1.2.1.ii). The buffer was degassed and filtered before applying to the column.

ii) **Preparation of washing buffer (100mM sodium acetate-acetic acid buffer pH 5.5 containing 1mM $MnCl_2$, 1mM $CaCl_2$ and 100mM NaCl)**: As the binding buffer and the washing were same, washing buffer was also prepared in the same way as the binding buffer. The buffer was degassed and filtered before applying to the column.

iii) Preparation of elution buffer (100mM sodium acetate-acetic acid buffer pH 5.5 containing 400mM dextrose (glucose)): 6.80 g of sodium acetate trihydrate ($NaC_2H_3O_2.3H_2O$) was dissolved in about 450 ml of deionized distilled water in a 500 ml beaker. 36 g of dextrose was also dissolved in the solution. (Weichselbum *et al*., 1946)

Before adjusting pH of the buffer, the pH meter was calibrated from pH 4 to pH 7. pH of the solution was adjusted to 5.5 with acetic acid. The final volume was made 500 ml in a volumetric flask adding deionized distilled water. The buffer was degassed and filtered before applying to the column.

1.2.6.2 Purification of lectin

The clear protein solution obtained after dialysis followed by centrifuge was injected to the column packed with Sephadex G 200 gel. The column was washed with washing buffer (100mM sodium acetate-acetic acid buffer pH 5.5 containing 1mM $MnCl_2$, 1mM $CaCl_2$ and 100mM NaCl) to wash out the unbound protein from the column. Washing was continued until the absorption at 280nm wave length and 0.02 range in the Bio-mini UV monitor became linear. After washing, the bound protein was eluted with elution buffer (100mM sodium acetate-acetic acid buffer pH 5.5 containing 400mM dextrose).

Figure 1-6: Operation of high performance liquid chromatography (HPLC).

1.2.7 Removing dextrose from affinity purified protein by dialysis

The affinity purified protein solution was dialyzed in 10 kDa dialysis bag against deionized distilled water. Dialysis was carried out with continuous stirring to enhance the process at 4-6^{o}C. After some hours of stirring the water was replaced by fresh water. This was done for several times. The presence or absence of dextrose was detected by dextrose test kit (Trinder J., 1969; Teuscher *et al* 1969). After dialysis the purified lectin was collected in a stoppered bottle.

1.2.8 Storage

1.2.8.1 Solution preparation

i) **Preparation of 20% sodium azide solution**: 2 gm of sodium azide was dissolved in 8 ml deionized distilled water. The final volume was made up to 10 ml by adding deionized distilled water.

ii) **Preparation of 20% ethanol solution**: 20 ml of ethanol was added in 80 ml of deionized distilled water. The solution was degassed and filtered and stored in a stoppered bottle.

1.2.8.2 Storage of medium

20% ethanol solution (three bed volume) as preservative was passed through the column. The medium was stored at 10-15^{o}C.

1.2.8.3 Storage of lectin

Short term storage: The volume of the purified lectin solution was measured by a measuring cylinder and 20% sodium azide solution was added to it to make the final concentration of sodium azide 0.1%. 0.1% sodium azide was used as preservative. The lectin solution was stored at 4-6^{o}C.

Long term storage: The purified lectin solution was taken in round bottom flask and iced in deep freeze with continuous spinning. As a result, the surface area of ice formed on the internal wall of the flask was increased which facilitate rapid removal of water during lyophilyzation. The iced lectin was lyophilized with the help of a freeze dryer. After that the lyophilized lectin was preserved in deep freeze.

1.2.9 Test of purity

1.2.9.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method

Principle: Polyacrylamide gel electrophoresis method is commonly used for checking the purity of proteins and their molecular weight determination. Sodium dodecyl sulfate (SDS) is anionic detergent that binds to most proteins in amounts roughly proportional to molecular weight of protein, about one molecule of SDS for every two molecules of amino acid residues. The bound SDS contributes large net negative charge, rendering the intrinsic charge of the protein insignificant. In addition, the native conformation of the protein is altered when SDS is bound and most protein assumes similar shape and thus similar ratio of charge to mass. Slab gel electrophoresis in presence of SDS therefore separates proteins almost exclusively on the basis

of mass, with smaller polypeptides migrating more rapidly. Protein-SDS complexes will therefore all move towards the anode during electrophoresis and their movements are inversely proportional to their molecular weights. If standard proteins of known weights are also run, the molecular weights of sample proteins can be determined by comparing them with proteins of the known molecular weights. The protein pattern of the selected fractions was determined by 10% SDS-PAGE according to the method of Laemmli (1970).

1.2.9.2 Reagents and solutions

i) **Preparation of 50% TCA (Trichloroacetic acid) solution**: 25 gm of TCA was dissolved in about 30 ml of deionized distilled water in a 50 ml volumetric flask. The final volume was made up to the mark by adding deionized distilled water.

ii) **Preparation of 30% acrylamide solution**: 14.5 gm acrylamide and 0.5 gm N,N′-methylene-bis-acrylamide were dissolved in 35 ml of deionized distilled water in a 50 ml volumetric flask and the final volume was made up to the mark by adding deionized distilled water. The solution was filtered and stored in a dark bottle at room temperature.

iii) **Preparation of 1.5 M Tris-HCl buffer (pH 8.8)**: 18.15 gm of Tris base was dissolved in 90 ml of deionized distilled water in a conical flask and mixed well. The pH of the solution was adjusted to 8.8 by adding concentrated HCl. The final volume was made up to 100 ml with deionized distilled water.

iv) **Preparation of 0.5 M Tris-HCl buffer (pH 6.8)**: 0.5 M Tris-HCl was prepared by dissolving 6.05 gm of Tris base in about 90 ml of deionized distilled water. After adjusting the pH to 6.8 with concentrated HCl the final volume was made up to 100 ml with deionized distilled water.

v) **Preparation of 0.75 M Tris-HCl buffer (pH 8.0)**: 0.75 M Tris-HCl was prepared by dissolving 9.08 gm of Tris base in about 90 ml of deionized distilled water. After adjusting the pH to 8.0 with concentrated HCl, the final volume was made up to 100 ml with deionized distilled water.

vi) **Preparation of 20% SDS (sodium dodecyl sulfate)**: 20% SDS was prepared by dissolving 10 gm of SDS in 40 ml of deionized distilled water. The final volume was made up to 50 ml with deionized distilled water.

vii) **Preparation of 10% ammonium persulfate (APS)**: 10% APS was prepared by dissolving 0.5 gm APS in 4 ml of deionized distilled water. The final volume was made up to 5 ml with

deionized distilled water. The solution was stored in eppendorf tubes (500 µl in each tube) at 10-15°C.

viii) **1 mg/ml Bromophenol blue solution**: 1 mg of bromophenol blue solution was prepared by dissolving 1 mg bromophenol blue in 1 ml deionized distilled water.

ix) **TEMED**: The commercially available TEMED from Sigma Chemicals Co., USA was used without modification.

x) **Preparation of sample buffer**: The sample buffer was prepared by mixing the components as given below and was stored at 10-15°C.

Components	**Amount**
0.5 M Tris-HCl buffer, pH 6.8	1.0 ml
Glycerol	0.5 ml
20% SDS	0.5 ml
2-Mercaptoethanol	0.25 ml
1 mg/ml Bromophenol blue	0.05 ml
Deionized distilled water	2.2 ml
Total	**5.0 ml**

This made the solution of following compositions 0.1 M Tris HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.01 mg/ml bromophenol blue.

xi) **Preparation of electrophoretic buffer**: 1000 ml of electrophoretic buffer was prepared by mixing the components as given below.

Components	**Amount**
Tris base	6.0 gm
Glycine	28.8 gm
20% SDS	5 ml
Deionized distilled water to	1000.0 ml

xii) **Preparation of coomassie brilliant blue (CBB) staining solution**: It was prepared by mixing the following components.

Components	**Amount**
CBB R 250	0.5 gm
Methanol	250 ml
Glacial acetic acid	25 ml
Deionized distilled water	225.0 ml
Total	**500.0 ml**

xiii) **Preparation of CBB destaining solution**: The CBB destaining solution was prepared by mixing the components as given below.

Components	**Amount**
Methanol	100 ml
Glacial acetic acid	100 ml
Deionized distilled water	800 ml
Total	**1000.0 ml**

1.2.9.3 Sample preparation

100 µl aliquot of the protein sample was mixed with sample buffer (1:1, v/v) in an eppendorf tube and heated for 5 min at 100°C. The sample was then used for SDS-PAGE.

1.2.9.4 Preparation of the markers proteins

100 µl marker protein and 1 ml of sample buffer was added to the vial. It was mixed well by inversion and then on a vortex mixture for about 5 seconds to complete solubilization. The aliquot was stored at -20°C or below. The aliquot was incubated in a boiling water bath for 60 seconds immediately before application of the markers to gel. 10 µl was applied for a standard size gel (16 cm × 14 cm) and 5 µl for mini size gel (10 cm × 10 cm). The following proteins were used as markers-

Proteins	**Approximately molecular weight**
Ovalbumin	45 KDa
Carbonic anhydrase	29 KDa
Tripsin inhibitor	20 KDa
Lysozyme	14.6 KDa

1.2.9.5 Procedure for SDS-PAGE

Clean and dry plates (10 cm ×10 cm) were assembled with a spacer (1 mm thick) and were held together on a gel casting stand. The assembly was checked for leakage.

Preparation of separating gel: The separating gel was prepared by mixing the following components.

Components	**12.5% Separating gel**
Deionized distilled water	9.83 ml
30% Acrylamide	12.38 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml
20% SDS	150 µl
10% APS	150 µl
TEMED	15 µl

The freshly prepared separating gel was poured between the glass plates carefully to avoid the inclusion of air bubbles. The upper level of the separating gel was overlaid with deionized distilled water or isopropanol to create a uniform upper level. It was kept undisturbed for 30 minutes.

Preparation of stacking gel: The stacking gel was prepared by mixing the following components.

Components	**Stacking gel**
Deionized distilled water	2.5 ml
40% Acrylamide	1.7 ml
0.5 M Tris-HCl, pH 6.8	5.7 ml
20% SDS	50 µl
10% APS	100 µl
TEMED	9 µl

Before pouring the stacking gel, deionized distilled water was poured off. After pouring the stacking gel, a 13 well comb was inserted carefully. After about 30 minutes the comb was gently removed. The portion, that did not polymerize, was removed cautiously.

The samples along with standard markers were loaded in the well carefully avoiding cross contamination. The samples were then overlaid with electrophoresis buffer. A constant current (30 mA) was then applied. When the sample reached to the bottom of the gel, the electricity supply was disconnected and gels were removed from the glass plates and were kept immersed in freshly prepared staining solution for 2-3 hours with gently shaking. It was then kept immersed in freshly prepared destaining solution with gentle shaking till the gel background became transparent.

1.2.10 Molecular weight determination by SDS-PAGE method

The molecular weight of the purified protein was determined by SDS-PAGE method. The purified protein was run through electrophoresis gel with several marker proteins. After running the purified protein through the electrophoresis gel, the molecular weight of the protein was calculated with respect to the marker proteins.

1.3 Results and Discussion

Lectins are widely used in many sectors such as medicine, warfare and analytical biochemistry especially for the purification of glycoproteins *e.g.* peroxidase. Most of the lectins used for commercial purification of peroxidase are collected from jack bean. This jack bean does not grow well in Bangladesh and commercially available jack bean lectins are expensive too. Based on previous screening experiment on various Bangladeshi plant species in our laboratory it has been found that, sword bean contains considerable amount of lectin. Hence, it is worthwhile to develop an efficient, simple and economical method for the purification of lectin from sword bean. Various affinity media have been widely used for the separation of lectins in the final step in the multistep protocols. The present work shows a simple and inexpensive method for the affinity purification of sword bean lectin in a single step.

1.3.1 Preparation of protein sample for purification in affinity column

1.3.1.1 Extraction and dialysis

For protein extraction, delipidated sword bean meal was taken in 100mM sodium acetate-acetic acid buffer pH 5.5 (1:5). Extraction was continued for 5 hours at 4-6°C temperature with continuous stirring. The protein solution was then separated from the slurry by centrifuge at 11,000 rpm for 10 minutes at 4-6°C. The solution was subjected to 90% ammonium sulfate precipitation overnight at 4-6°C. After centrifuge, the precipitate was dissolved in 100mM sodium acetate–acetic acid buffer pH 5.5 containing 1mM $MnCl_2$, 1mM $CaCl_2$ and 100mM NaCl. After that the clear solution was dialyzed in 10 kDa dialysis bag against the same buffer at 4-6°C temperature to remove ammonia. The presence of ammonia was detected by barthelot method. Dialysis was carried out three times with fresh buffer. When the presence of ammonia in the dialysate was negligible, the protein solution was taken out from the dialysis bag and centrifuged to get clear solution. This impure protein solution was ready to apply to the affinity column.

1.3.1.2 Chromatogram of affinity purification

When impure protein solution was injected into the affinity column packed with Sephadex G-200 gel, the following chromatogram was obtained. In the chromatogram, horizontal axis indicates time in minutes and vertical axis indicates detector response in mV at 280 nm.

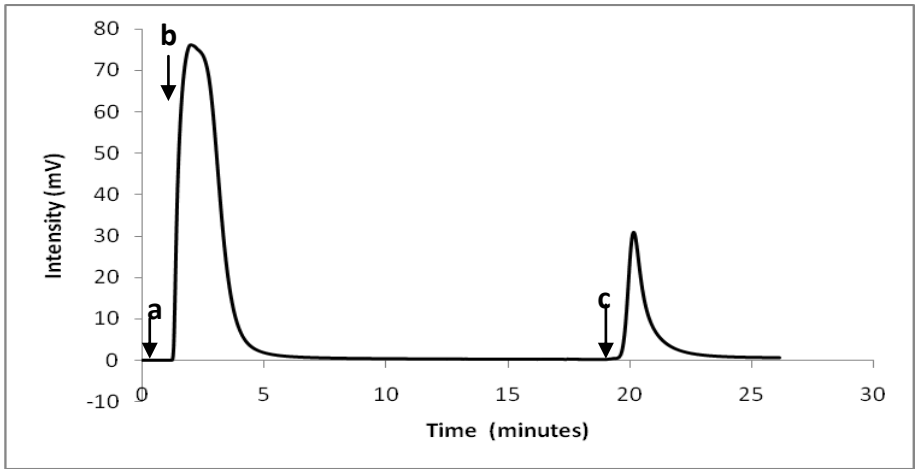


Figure 1-7: Chromatogram of affinity purification, (a) Equilibration with 100mM sodium acetate-acetic acid buffer pH 5.5 (b) Injection of 6 ml crude extract and washing with 100mM sodium acetate-acetic acid pH 5.5 containing 1mM $MnCl_2$, 1mM $CaCl_2$ and 100mM NaCl (c) Elution of bound protein with 100mM sodium acetate-acetic acid pH 5.5 containing 400mM dextrose.

In the chromatogram, the large peak indicates the removal of contaminant proteins during washing the affinity column. When the response in the detector became linear, the matrix-bound protein was eluted as indicated by the small peak.

1.3.1.3 Dialysis

The eluted solution contains dextrose, salts and protein molecules. To remove adhere salts and glucose from the protein solution; this was dialyzed against deionized distilled water. Dialysis tubing was activated by boiling in 250 ml solution containing 5 g Na_2CO_3 and 0.0931 g EDTA disodium salt for 10 min. followed by boiling in 250 ml distilled water. The tube was then knotted carefully at one end. The concentrated protein solution was placed in dialysis bag, and the other end was knotted before dialysis started. Then the dialysis bag was placed in a large volume of buffer and stirred for 12 hours, which allows the solution inside the bag to equilibrate with the solution outside the bag with respect to salt concentration. When this process of equilibration was repeated several times, the protein solution in the bag was reached a low salt concentration. The protein solution thus obtained was designated as purified protein. The purity of the protein was checked by SDS-PAGE.

Figure 1-8: Photograph of dialysis process.

1.3.1.4 UV-Vis spectrum of purified potein

A protein consists of an amide (peptide) backbone with various side chains on the α-carbons between each amide. The dominant chromophore is the amide group, which has a weak nπ* transition at about 220 nm and an intense ππ* transition at about 195 nm. The elecronic transitions of most side chains occur bellow 200 nm, and are overpowered by intense ππ* of the amides. Exceptions are phenylalanine, tyrosine, tryptophan, cysteine, methionine, and disulfide groups, which begin their electronic transitions just below 300 nm. The π systems of phenylalanine, tyrosine, and tryptophan have the ππ* transitions. We are primarily interested in the spectra of affinity purified protein and the result has been presented in figure 1-9. The spectrum clearly indicates that the affinity purified protein consists of considerable amount of aromatic amino acids *e.g*. phenylalanine, tyrosine, tryptophan etc.

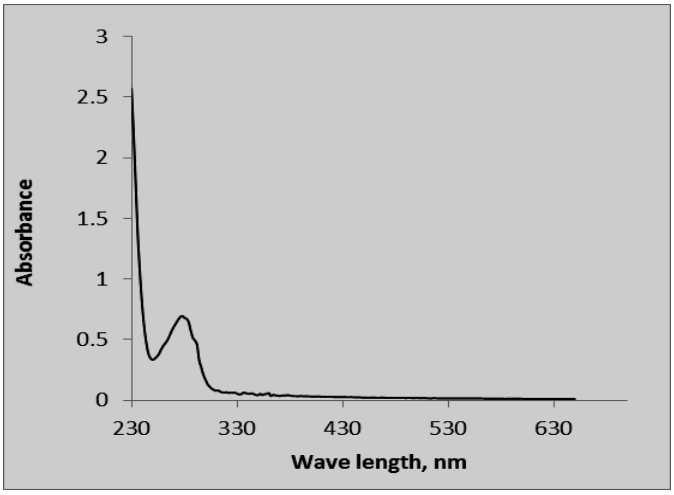


Figure 1-9: UV-Visible spectrum of purified protein.

1.3.1.5. Determination of the protein content by Biuret method

20 µl protein sample and 20 µl standard protein sample were added to 1.0 ml of total protein reagent in different test tubes, the mixtures were shaken for few times, allowed to react for 10 min at room temperature. The absorbance of sample and standard against the reagent blank were measured within 30 min at 546 nm using a UV-Vis Spectrophotometer. The amount of protein content was determined using the following equation.

$$C = 80 \times \frac{\Delta A_{sample}}{\Delta A_{standard}} [mg/ml]$$

It was found that 10 g seed contains about 1.34 g of protein among which about 0.23 g is Sephadex binding lectin and the remaining 1.06 g is other protein. Thus, the approximate ratio of Sephadex binding lectin and other proteins in sword bean is 2:9.

Table 1-1: A summary of the purification of lectin from sword bean by Sephadex affinity chromatographic technique.

	Volume (ml)	Absorbance at 546 nm	Protein content (mg/ml)	Total protein (mg)	% Protein
Standard		0.41	80		
Crude extract	6	0.143	27.9	167.4	13.392
Unbound	32	0.021	4.1	131.2	10.66
Bound	11	0.011	2.15	23.65	2.365

1.3.1.6 Test of purity by SDS-PAGE

Purity of the bound fraction from the affinity column was checked by SDS-PAGE analysis, performed under reducing conditions, on a 1 mm separating gel in a final concentration of 12.5% of acrylamide-bisacrylamide solution. About 7 µg of total proteins were analyzed by performing the electrophoresis. Detection of the protein bands was performed with the Coomassie Brilliant Blue R-250 staining method and degree of purity was determined by electronic scanning.

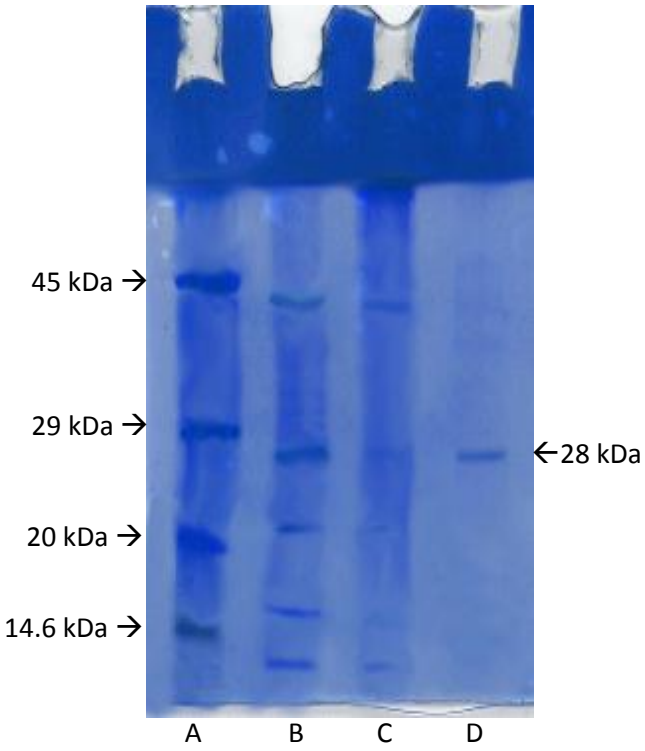


Figure 1-10: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify individual proteins present at various stages of the purification. A= Marker protein, B = Crude extract, C= Flow through and D= Affinity purified protein.

The scanning result has been presented in figure 1-10. In figure, lane B indicates the presence of five kinds of protein in crude extract. However, the presence of single band as shown in lane D indicates that, Sephadex affinity matrix system was able to purify only one kind of protein in a single step. Thus, Sephadex affinity matrix system at pH 5.5 was proven to be a useful tool in the purification of glucose-binding protein in a single step.

1.3.1.7 Determination of molecular weight of lectin by SDS-PAGE

The molecular weight of the affinity purified lectin was determined by SDS-PAGE using Ovalbumin (45 kDa), Carbonic anhydrase (29 kDa), Trypsin inhibitor (20 kDa) and Lysozyme (14.6 kDa) as reference protein. The molecular weight was calculated from the standard curve of reference proteins which was constructed by plotting $\log_{10}$ molecular weight against relative

mobility of the proteins on gel after electrophoresis and the molecular weight of the purified protein was found to be 28 kDa (Figure 1-11).

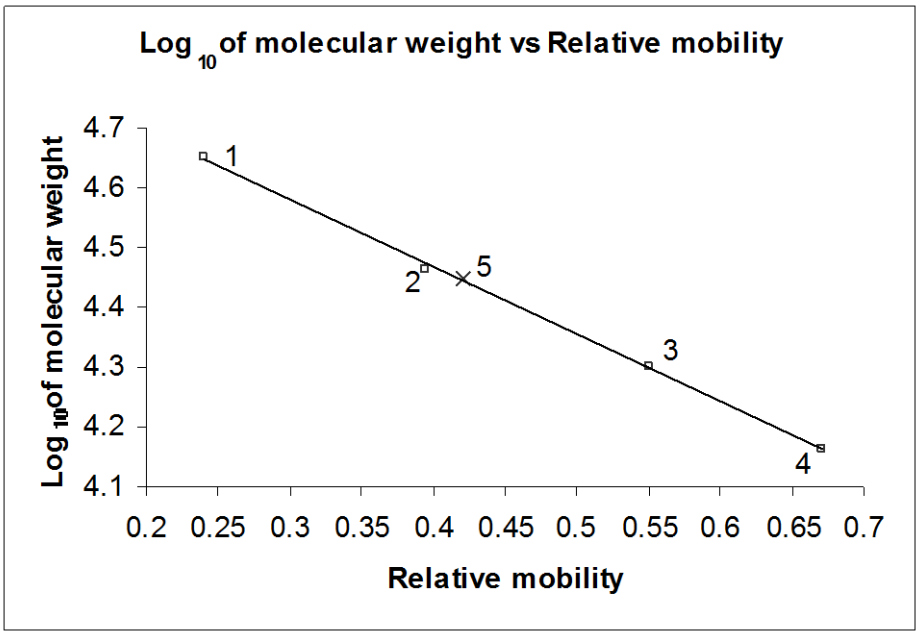


Figure 1-11: Estimation of molecular weight of the purified lectin derived from sword bean. Relative mobilities of marker proteins (labeled l–4) were used to obtain the standard curve as described by Shapiro et al. (1967) and Weber and Osborn (1969). 1, 2, 3, and 4 were standard markers of molecular weight 45 kDa, 29 kDa, 20 kDa and 14.6 kDa respectively and 5 denote the purified sword bean lectin.

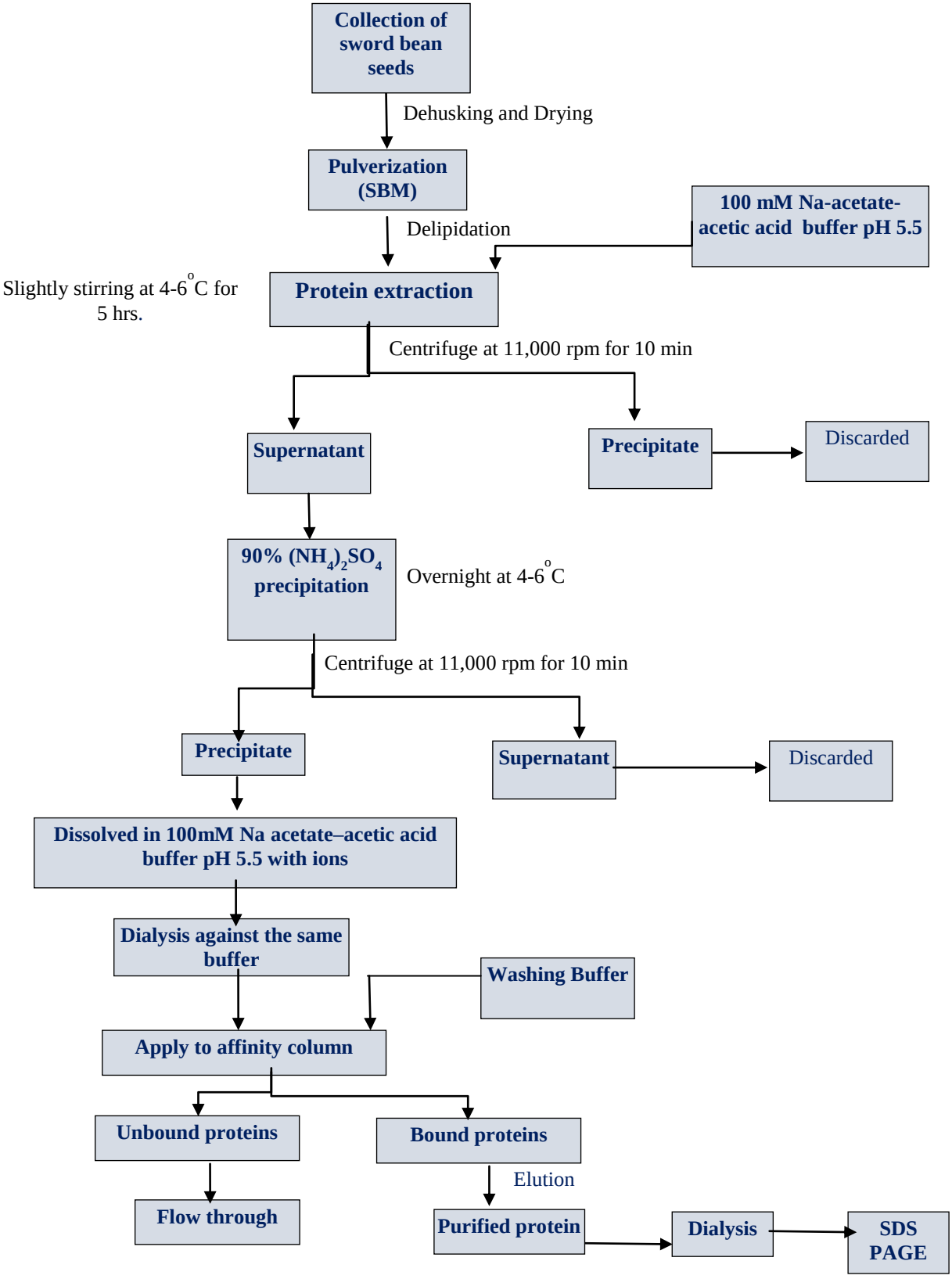
1.4 Conclusion

Lectins derived from plants shows different types of biological activity. Such as cell agglutination, toxicity, mitosis and cell growth inhibition. Plant lectins are more useful than animal lectins. Recently some studies on animals has proved that *Abrus precatorius* agglutinin which is a ribosome inactivating lectin inhibit tumor growth in nude mice bearing xenografts of human hepatoma HepG2 cells (Mukhopadhyay *et al.*, 2014). A previous screening programme showed that sword bean contain considerable amount of lectin. In our country sword bean plant is now cultivated in different area. To isolate and purify sword bean lectin (SBL) efficiently and in a single step we used Sephadex G-200 gel as affinity matrix. SBL was extracted from delipited sword bean meal using sodium acetate-acetic acid buffer pH 5.5.The centrifuged

sample was subjected to dialysis process to remove excess ammonium sulfate. Salt free SBL was applied to affinity column and purified SBL was collected by elution. From 10 g seeds about 1.34 g of protein was extracted among which about 0.23 g is Sephadex binding lectin and the remaining 1.06 g is other protein. Thus, the approximate ratio of Sephadex binding lectin and other proteins in sword bean is 2:9.

Using 12.5% SDS-PAGE gel purity of the affinity column bound fraction was checked. Comparing with four reference protein, Ovalbumin (45 kDa), Carbonic anhydrase (29 kDa), Trypsin inhibitor (20 kDa) and Lysozyme (14.6 kDa) and from the standard curve of reference proteins which was constructed by plotting $\log_{10}$ molecular weight against relative mobility of the proteins on gel after electrophoresis the molecular weight of the purified SBL was found to be 28 kDa. So it can be concluded that the affinity matrix system purified sword bean lectin in a single step which can be used as molecular tool in different discipline of medicine and biology.

1.5 Flow chart for isolation and affinity purification of sword bean lectin



2.1 Introduction

2.1.1 Cancer

Cancer is the name of a group of diseases which is characterized by uncontrolled growth and spread of abnormal cells. If the growth is not controlled, it can result in death. External causes of cancer are tobacco, chemicals, radiation and infectious organisms. Internal factors are inherited mutation, hormone, immune condition and mutation that occur from metabolism. Ten or more years often passed between exposures or mutation and detection of cancer. Treatment of cancer can be done by surgery, radiation, chemotherapy, hormone and immunotherapy. In spite of its property that cancer affect people at all ages, even fetuses, the risk increases with age (Cancer Res. UK, 2007). Cancer causes about 13% of all deaths (WHO, 2007). In economically developed countries cancer leads the causes of death which took second place in developing countries (World health organization; 2008). Cancer can affect all animals. In the U.S. and other developed countries, cancer is presently responsible for about 25% of all deaths (Jemal *et al.*, 2005). On a yearly basis, 0.5% of the population is diagnosed with cancer. The statics below are for adults in the United States and may vary substantially in other countries.

Table 2-1: Different types of male and female cancer in United States.

Male		Female	
Most common (By occurrence)	Most common (By mortality)	Most common (By occurrence)	Most common (By mortality)
Prostate cancer (33%)	Lung cancer (31%)	Breast cancer (32%)	Lung cancer (27%)
Lung cancer (13%)	Prostate cancer (10%)	Lung cancer (12%)	Breast cancer (15%)
Colorectal cancer (10%)	Colorectal cancer (10%)	Colorectal cancer (11%)	Colorectal cancer (10%)
Bladder cancer (7%)	Pancreatic cancer (5%)	Endometrial cancer (6%)	Ovarian cancer (6%)
Cuteneous Melanoma (5%)	Leukemia (4%)	Non-Hodgkin Lymphoma (4%)	Pancreatic cancer (6 %)

Cancer poses serious health problems both in developed and developing countries. The prevention and control of cancer in developed countries deserve urgent attention since the

diseases is expected to double in these countries in the next 20 to 25 years. Though cancer is a genetic disease, the problem of cancer in Bangladesh is particularly acute because of poverty, illiteracy and other diseases associated with poor nutrition and lack of basic knowledge of people about health matters.

2.1.2 Cancer condition in Bangladesh

Bangladesh is the 9th most populous country of the world where 142 million people live. The country has 13 to 15 lakh cancer patients, with about 2 lakh patients newly diagnosed with cancer each year (Uddin *et al.*, 2013, Noronha *et al*., 2012). According to cancer registry report of National Institute of Cancer Research and Hospital 2005-2007, the prevalence rates of the leading cancers in both males and females are presented in Tables 2-2.

Table 2-2: Leading cancers and the prevalence in last five years in males

Cancer Type	Prevalence in last five years (%) (male)	Prevalence in last five years (%) (female)
Lung cancer	13.1	2.0
Lip and oral cavity cancer	11.9	6.5
Other pharynx	8.2	-
Colo-rectal cancer	6.5	2.7
Stomach cancer	4.7	1.8
Esophageal cancer	4.1	1.9
Non-Hodgkin lymphoma	4.7	1.3
Hodgkin lymphoma	2.2	0.8
Bladder	3.4	-
Prostate	2.3	-
Liver	1.5	0.6
Leukemia	0.6	
Breast	-	32.8
Cervical	-	26.1
Ovarian	-	3.3

2.1.3 Classification

Cancers are classified in two ways: by the type of tissue in which the cancer originates (histological type) and by primary site, or the location in the body where the cancer first developed. Among hundreds of different cancers five major groups are carcinoma, sarcoma, myeloma, leukemia and lymphoma from the histological point of view. In addition, there are also some cancers of mixed type.

2.1.4 Development of cancer

Cancer develops by the multistage process where elderly are more susceptible than other. This may take 15-45 years before clinical signs are shown. Cancerous growth forms as the result of a sequence of events over a period of time. The events are associated with the exposure of DNA to harmful molecules originating from, within or outside of the body. An average of five or six chemical insults responsible for the change of genes are suggested by researchers which lead to cancer. People who may not have cancer preventing enzymes or who have enzymes prone to contribute cancer formation are genetically susceptible to cancer.

There are three phases of development involved in the formation of cancerous growth.

- In the first stage mutation of DNA occurs and DNA repair (by enzymes) is not possible or undergoes faulty DNA repair.
- The second step involves the promotion of uncontrolled growth and proliferation of mutated cells. After some time these cells lose their normal abilities and only reproduce.
- In third step metastasis formed which is the invasion of cancerous cells into nearby tissues. The cancerous cells migrates to other tissues via circulatory or transport system.

Normal healthy cells grow and differentiate in a very orderly and well controlled way. In normal tissues, there is a balance between cell proliferation, growth arrest and differentiation as well as loss of mature cells by programmed cell death. When this homeostatic control mechanism failed then tumors develop which means the appropriate number of cells in normal tissues is lost or disturbed leading to an imbalance between cell proliferation and cell death.

2.1.5 Causes of cancer

Cancer is a diverse class of diseases which differ widely in their causes and biology. The common thread in all known cancers is the acquisition of abnormalities in the genetic material of the cancer cell and its progeny. Research into the pathogenesis of cancer can be divided into three broad areas of focus. The first area of research focuses on the agents and events which cause or facilitate genetic changes in cells destined to become cancer.

2.1.6 Treatment of cancer

Cancer can be treated depending upon the location and grade of the tumor and the stage of the diseases by surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy. The treatment procedure is also dependent on the general state of the patient (performance status). A number of experimental cancer treatments are also under development. Complete removal of the cancer without damage to the rest of the body is the goal of treatment. Sometimes this can be happened by surgery, but the tendency of cancers to invade adjacent tissue or to spread to distant sites by microscopic metastasis often limits its effectiveness. The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body. Radiation can also cause damage to normal tissue. Because "cancer" refers to a class of diseases, it is unlikely that there will ever be a single "cure for cancer" any more than there will be a single treatment for all infectious diseases (Murphy *et al.*,1997; Simone *et al.*, 2000).

2.1.7 Lectins as an alternative for cancer treatment

Lectins are proteins or glycoproteins from non-immune origin that specifically recognize cell surface molecules with at least two binding sites to carbohydrates (hence their ability to agglutinate cells), precipitating the corresponding glycoconjugates. They are found in all kinds of organisms, including animals, plants, fungi, bacteria and viruses (Lotan & Raz 1988, Wang 1996). Lectins have a wide spectrum of functions related to them shown in Figure 2-1 (Sharon & Lis 1989).

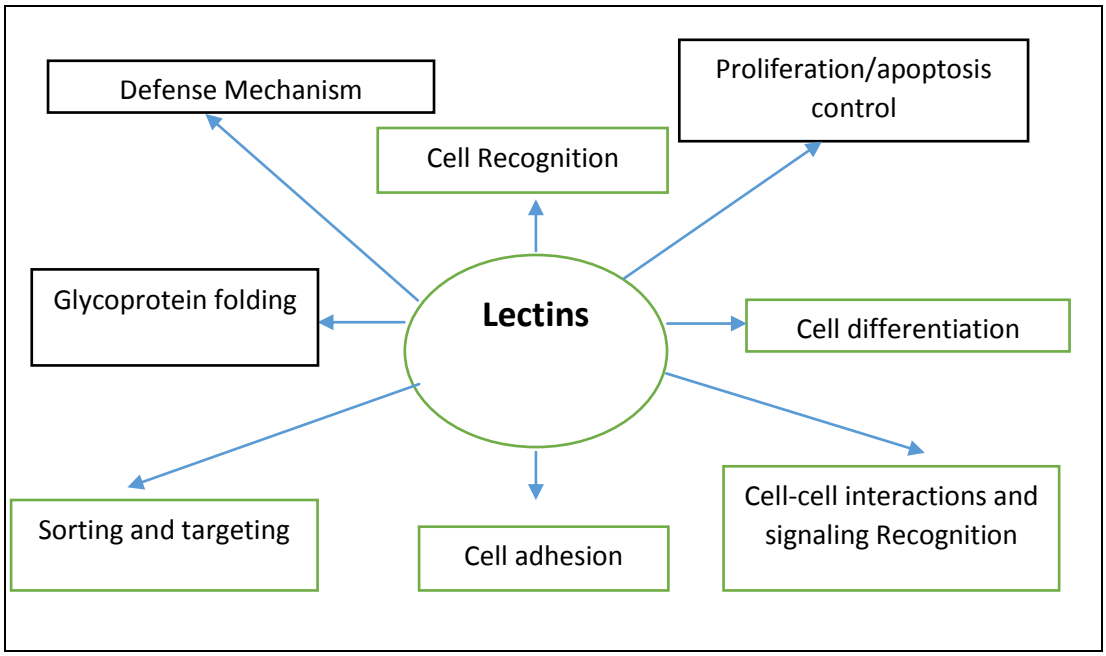


Figure 2-1: Some biological functions of lectins in live organisms (Sharon and Lis 1989, Baronde 1981)

Lectins have anti-cancer properties *in vitro* and *in vivo*, preferentially binding to cancer cell membranes or their receptors and undergo cytotoxicity, apoptosis, autophagy and inhibition of tumor growth (Gonzalez & Prisecaru 2005). Antitumor effect and anticarcinogenic activity of lectins are due through different mechanisms as the induction of remission in certain tumors, having a direct anti-tumor cytotoxic effect, by improving the antineoplastic effect of radiation and chemotherapy, by promoting restoration of normal growth in cancer cells, by amplifying the immunogenicity of tumor cells and because of their differential cytotoxic effect on malignant cells with respect to normal cells, they exhibit minimal risk of anti-tumor cytotoxic activity (Ruiz *et al.*, 2002).

2.1.8 Aim of the present work:

Lectins, the heterogenous group of proteins of non-immune origins display a variety of biological activities. They also bind reversibly to mono and oligosaccharides with high specificity but without any catalytic activity (Lis & Sharon 1998) and are highly distributed in nature. Most of the lectins are purified from plant sources. The main sources of lectins in plants are mature seeds, fruits, leaves.

Lectins serve many different biological functions, from the regulation of cell adhesion to glycoprotein synthesis and the control of protein levels in the blood. They also display anti-tumor (Pusztai *et al* 1998), immunomodulatory (Abdullahev and Mejia 1997), anti-fungal (Rubinstein *et al.,* 2004) and anti-insect (Barrientos and Gronenborn 2005) activities. It has been long known that these proteins are capable of agglutinating red blood cells in mammals, including all human blood types (Felsted *et al.,* 1981). Thus, the purified lectins are important in a clinical setting because they are used for blood typing. Some of the glycolipids and glycoproteins in an individual's red blood cells can be identified by lectin. Other functions are its ability to bind to the surface of the intestinal walls. They have also been associated with the appearance of lesions, disruptions and abnormal growth of the microvilli, as well as the malabsorption of nutrients across the membranes (Chrispeels *et al.,* 1997). Recent studies have shown that these compounds possess anti-human immunodeficiency virus (HIV) activity (Herre *et al*., 2004). In analytical biochemistry, lectins are used as a tool for affinity purification of glycoproteins *e.g.* glucose oxidase, peroxidase etc.

A group of disease known as cancer, is very much aggressive, invasive and sometimes metastatic may affect people at all ages. Apart from human it may affect other animal and plants (Tannock & Hilp, 2007). Different methods exist for the treatment of cancer in modern medicine including chemotherapy, radiotherapy and surgery. Among them chemotherapy is now considered as the most effective method of cancer treatment. But chemotherapeutic drugs are not devoid of their own intrinsic problems. Currently available chemotherapeutic agents insidiously affect the host cells, especially bone marrow, epithelial tissues, reticulo-endothelial system and gonads whereas an ideal anticancer agent should be both tissue and cell specific i.e. it should kill or incapacitate cancer cells without causing excessive damage to normal host cells (Mascarenhas, 1994). As the toxicity of chemotherapeutic drugs creates a significant problem in the treatment of cancer, various therapies have been propounded for the treatment of cancer. Some of which use plant derived products. Plants are reservoir of natural chemicals which have chemoprotective potential against cancer. A number of compounds from medicinal plants with potential anti-cancer activities has been reported recently (Taneja & Qazi 2007). Approximately 40% of modern drugs may directly or indirectly be related to natural compounds. Several plant-derived compounds have been approved as anticancer drugs – vinblastine, vincristine, etoposide, teniposide, taxol, taxotere, topotecan and irinotecan. In the other hand adequate drugs are not currently available to treat major solid tumors such as those of the lung, breast, prostate and colon. So there is an urgent need to find an appropriate treatment for the millions of patients who are affected (Fulder 1996).

It is known that conventional chemotherapeutical drugs are effective in their anti-cancer activity. But their high toxicity limits their clinical efficacy. Vectorization of the cytotoxic drugs only to the targeted cells is believed to be a feasible approach to circumvent the side-effect to some degree. Nanoparticulate delivery system is believed to increase the stability of the encapsulated or conjugated therapeutical molecule, improve the efficacy and alleviate the undesired side-effect.

The aim of our present investigation is to find the

1. **Hemagglutination studies of the purified lectin.**
2. **Antiproliferative activity of sword bean lectin and carbonate apatite against Ehrlich Ascites Carcinoma cell.**

2.1.9 Literature review:

Relatively few lectins have been purified from plants. Only recently have much attention been given to the isolation, determination of content and properties of legume root lectins (Horejsi *et al., 1978;* Gatehouse *et al* 1980; Quinn and Etzler 1987). As reported earlier, a lectins known as trifoliin has been isolated from the root of white clover (*Trifolium repens*) (Lis and Sharon 1986; Sharon and Lis 1989). This root lectin was purified and was found to be similar in immunological and electrophoretic properties to white clover seed lectin (Dazzo *et al.,* 1978). Also, roots of soybean (*Glycine max*) have hemeagglutinating activity and contain a protein which reacted with antibodies directed against soybean seed lectin. Thus, it was then assumed that seed lectin was the same as root lectin (Gatehouse *et al* 1980). However, more recent studies have revealed that this was not the case (Gatehouse *et al* 1980; Quinn and Etzler 1987).

The difference between root lectins and seed lectins was demonstrated through tests on the specificity of root lectins binding to *Rhizobia* conducted by Bohlhool and Schmidt, 1974, Chen and Phillips, 1976, Dazzo and Brill, 1977, Law and Strijdom, 1977 and Bhuvanesurai and Bauer (1978). As reviewed by Gatehouse and Boulter (1980), root lectin from *Pisum sativum* (pea) gave a different banding pattern in isoelectric focusing as compared to its seed lectin (Gatehouse *et al* 1980). In addition, the two lectins gave different carbohydrate specificities when assayed using sugar inhibition (Gatehouse *et al* 1980). Quinn and Etzler (1987) pointed out differences in amino acid composition between the root and seed lectins from *Dolichos biflorus* (horsegram). They also discovered differences in the banding patterns of the two lectins in isoelectric focusing.

Landsteiner's discoveries of human isoagglutinins in 1900 in Vienna and the species specificity of plant agglutinins as well as his comparison of haemagglutinating/haemolyzing activities with natural antibodies led W. C. Boyd "to test seeds for blood group specificity" (Boyd 1963). With extracts of the lima bean (*Phaseolus lunatus limensis*, Leguminosae) he found A-type specificity and proposed "the term 'lectin' (from the Latin *legere*, to choose or to pick out) for these and other antibody like substances" (Boyd and Shapleigh 1954). Drawing on the competitive inhibition of antibody-antigen reactions by compounds structurally related to the haptenic group described by Landsteiner and van der Scheer (1931) and the binding of con A to sugar compounds documented by Sumner and Howell (1936), inhibition of haemagglutination mediated by eel (*Anguilla anguilla*) serum and extracts of *Lotus tetragonolobus* seeds by Fuc and of haemagglutination by other plant lectins by GalNAc provided first insights into the chemical nature of the blood group determinants (Morgan and Watkins 1953). From this starting point, the chemical nature of the blood group substances as oligosaccharides could be delineated. For the reaction of red blood cells with blood-group-specific lectins, the terminal *α*-linked monosaccharides GalNAc (group A), Gal (group B) and Fuc (group H) are decisive. It goes without saying that these studies were further extended thereafter and were crucial for "unravelling the biochemical basis of blood group ABO and Lewis antigenic specificity" (Morgan and Watkins 2000). With this focus on haemagglutination it is no surprise that the initial definition of the term *lectin* (Boyd and Shapleigh 1954) placed special emphasis on just this aspect. As apparent from the example of the homologous lectin subunits of the *Ricinus* agglutinin/toxin, however, strict application of the criterion of haemagglutination would separate related proteins.

Thus, haemagglutination or precipitation of glycans which depend on at least bivalency are now considered as being only one example of a broad panel of assays able to detect carbohydrate binding (Gabius 1998). Moreover, lectins ought to be distinguished from other molecules able to clump erythrocytes together.

Plant lectins attracted attractions because of their anticancer properties and potential application as antitumor agents. Due to widespread occurrence and relatively similar defensive properties a huge amount of research on plant lectins have been done. Plant lectins affect both apoptosis and autophagy by modulating representative signalling pathways involved in Bcl-2 family, caspase family, p53, PI3K/Akt, ERK, BNIP3, Ras-Raf and ATG families, in cancer (Jiang *et al*,. 2015). *Polygonatum odoratum* (POL) lectin categorized by Wu and Bao (2013) as part of the GNA related family which specifically bind to the monosacchaaride mannose due to specific amino acid sequence. This lectin might be linked to their anti-cancerous properties. POL has been

found to induce signs of apoptosis in A549 lung cancer cells without affecting healthy HELF lung cells. The inhibitory rate was almost 50% after incubating A549 cells for 24 hours when a concentration of 23µg/ml of POL was used and it was determined that apoptosis was induced by means of suppressing a mitochondrial-mediated pathway known as Akt-NF-ƙb pathway (Li, C.; *et al.*, 2014). Apoptosis and autophagy was triggered by POL in human MCF-7 breast cancer cells by targeting epidermal growth factor receptor-mediated Ras-Raf-MEK-ERK signaling pathway (Ouyang, L. *et al.*,2014). Lectin extracted from mistletoe plant species have been well studied due to their widespread effectiveness on a variety of neoplastic cells, yet it is one of the more controvertial lectins when regarding cancer treatment. Sometime this lectin shows pro-apotosis effects when certain dosages of mistletoe lectins are given, whereas other concentrations produce anti-apoptic consequences (Lyu and Park 2007).

Li. LN.; *et al.*, (2011) showed that Chinese mistletoe lectin-1 (CM-1) is an additional lectin that can induce apoptosis in colorectal cancer cells through down-regulation of miR-135 a&b expression and up-regulation of expression of the adenomatous polyposis coli (APC) gene leading to reduced activity of Wnt signaling, a gene downstream of APC. Wnt signaling controls β-catenin levels thus affecting gene expression and interference with this signal has been linked to 90% of colorectal cancer cases. Shi Z.; *et al.*,2014 showed that Concanavalin –A induces apoptotic morphology in cultured MCF-7 human breast carcinoma cell. Nude mice, bearing MCF-7 cell-derived tumors were injected intraperitonially with ConA (40mg/kg) daily for 14 days. As a result tumor volumes and weights decreased. In HeLa cells, Con A induced apoptosis and autophagic death through suppression of the phosphoinositol3-kinase/ Akt/ mammalian target of rapamycin pathway and promoted both autophagic and apoptic cell death through reactive oxygen species generation in HeLa cells (Roy, B.;*et al.*, 2014). A study was done with five different lectins: PHA (*Phaseolus vulgaris*), GSA (*Griffonia simplicifolia),* Con-A (*Concavalina A*), WGA (*Triticum vulgare*) and PNA (*Arachis hypogea)* on three colon cancer cell lines (Lovo, HCT-15 and SW837) showed that growth was affected in different ways depending on the concentration and type of lectin tested. So this could be concluded that those lectins have a potential to affect the growth of cancerous colonies *in vitro* (Kiss, R; *et al.*, 1997). Lectin from *Phaseolus vulgaris*, a common bean has mitogenic action on immune system cells and has the ability to specifically agglutinate malignant cells. This has developed a strong interest in research to use it as a treatment for tumor growth control (Riaño-Sánchez R., 1997).

Rapid progress in cell-targeted delivery has made great contribution in biomedical application, especially in cancer research. Nano- crystals loaded with therapeutic agents could elongate the

circulation time of drug in human body, accumulate anticancer drug at specific tumor sites and alleviate the potential side-effect of drug. Hence, we hypothesize that combination of nano-crystals with chemotherapeutic drug (natural/chemical) could present superior therapeutic effects to cancer with less side-effects arising from traditional anti-cancer drug.

Development of an efficient, safe nano-carrier system of carbonate apatite which has the ability to assist both intracellular delivery and release of DNA leading to very high level of trans-gene expression in cancer and and primary cells has been reported (Chowdhury E.H, *et al.* 2007, Chowdhury E.H, *et al.* 2006, Chowdhury E.H, *et al.* 2005]. Chua MJ *et al.*,2013 identified c-ROS1 as a highly promising therapeutic target through intracellular delivery of the target siRNA using carbonate apatite nanoparticles in presence of the traditionally used anti-cancer drugs.

2.2 Materials and Method

2.2.1 Haemagglutination studies of purified sword bean lectin:

a) Preparation of hemaglutination buffer (20 mM Tris-HCl buffer pH 7.5 containing 150 mM NaCl and 10 mM $CaCl_2$): 20 mM Tris-HCl was prepared by dissolving 0.24 gm of Tris base in about 90 ml deionized distilled water. Then 0.88 gm NaCl and 0.11 gm $CaCl_2$ were added. After adjusting the pH to 7.5 with concentrated HCl, the final volume was made 100 ml with deionized distilled water.

b) Preparation of 2% RBC suspension: Just before experiment, blood from chicken, mice, human blood types, bovine and goat were collected in a centrifuge tube containing sufficient amount of 1% NaCl solution. The blood samples were immediately centrifuged at 3000 rpm for 3 minutes. The supernatant were discarded and the cells were washed similarly for three times with the above solution. Finally a 2% suspension (w/v) of RBC was prepared in 1% NaCl.

c) Hemagglutination activity and biological specificity of lectin: The dialyzed protein was assayed for haemagglutinating activity. The hemagglutination was performed in U-bottom polystyrene microtiter plate: 50 µl of 20mM Tris buffer pH 7.5 containing 150mM NaCl and 10mM $CaCl_2$ were poured into A1 through C12. 50 µl of protein solutions in 20mM Tris-HCl buffer were added in A1 (crude extract), B1 (flow through) and C1 (purified protein) of the titer plate. The protein and buffer were mixed well. Two-fold serial dilution was carried out from 1:1 dilutions in wells A1, B1 and C1 down to 1:2048 dilutions in wells A12, B12 and C12. 50 µl of 2% cell solution was dispensed in well A1 to C12 each. A control containing 50 µl of 20mM

Tris buffer pH 7.5 containing 150mM NaCl and 10mM $CaCl_2$ and 50 µl cell suspensions were used in D1 to D4. The mixture in the titers plate was mixed well by gentle shaking with vortex mixture. The mixture was incubated at 37°C for an hour in order to allow for agglutination of the erythrocytes to take place. To ascertain biological specificity of SBL the hemagglutination activity was tested against chicken, mice, bovine, goat and human blood A,B,O groups' erythrocytes.

2.2.2 Hemagglutination Inhibition Studies:

a) Reagent: i) 1% NaCl solution, ii) hemagglutination buffer iii) 2% chicken RBC 1% NaCl iv) 200 mM of sugar solutions of D(+) Glucose, Maltose, D(-) Mannose, D(+) Galactose, L(+) Arabinose, D(-) Arabinose and Fructose.

b) Procedure: The hemagglutination inhibition test was performed in the presence of different sugars. The above mentioned sugars were dissolved in 20 mM Tris-HCl pH 7.5 containing 150 mM NaCl and 10 mM $CaCl_2$. 25 µl of hemagglutination buffer were added to the selected wells of the titre plate. Then 25 µl of sugar solutions were added to the first well of the titre plate and serially two-fold dilution was carried out down to12 well. Then 25 µl of protein solution (2.58mg/ml of sword bean protein) was added to every well of the titre plate. After 30 mins of incubation at room temperature, 50 µl of 2% chicken RBC's in saline were added to every well of the titre plate. A positive control containing i) 25 µl of lectin solution, ii) 25 µl of hemagglutination buffer, iii) 50 µl of 2% chicken RBC and a negative control containing i) 50 µl of hemagglutination buffer ii) 50 µl of 2% chicken RBC was also tested (Atkinson and Trust 1980). Then the plate was shaked by vortex mixture machine and further incubated at 37°C for 1 hr. Minimum inhibbitory concentration (MIC) was determined by serial dilution of the sugar solution. MIC was defined as the lowest concentration of sugar capable of complete inhibition of agglutination.

2.2.3 Antineoplastic studies: The antineoplastic study (*in vivo*) of the sword bean lectin against Ehrlich Ascites Carcinoma (EAC) cells was done in our laboratory using swiss albino mice.

2.2.3.1 Animal collection: Swiss albino mice of 5-7 weeks old, weighing 25±5 grams were collected from International Center for Diarrohoeal Disease Research, Bangladesh (ICDDR'B), Mohakhali, Dhaka.

Mice Vital Statics:

I. Scientific Name: Mus Musculus
II. Life Span: 2-3 years
III. Potential Life span: 4 years
IV. Desirable Environmental Temperature Range: 18-27°C
V. Desirable Relative Humidity Range: 30-70%
VI. Age at onset of Puberty: 28-40 days
VII. Estrus (heat) cycle length: 4-5 days
VIII. Estrus length (period during which female is receptive to male for copulation): 12 hours
IX. Gestation (pregnancy) period: 19-21 days
X. Weaving age: 21-28 days (Hamadeh and Ashrafi 2004)

2.2.3.2 Animal care:

a. Cage: Mice were kept in iron cages with staw dust and straw bedding which was changed regularly.
b. Temperature, light and humidity : The room temperature was maintained around 25-32ºC and a controlled 14 hours day light and 10 hours dark were maintained in the laboratory (animal house).
c. Food: Standard mouse diet (recommended and prepared by ICDDR'B) and water were given in adequate.

2.2.3.3 Experimental Tumor Model: Transplantable tumor EAC cells were used in this experiment. The initial inoculum of EAC cells was kindly provided by the Indian Institute of Chemical Biology (IICB), Kolkata, India. The EAC cells were thereafter propagated in our laboratory biweekly through intraperitoneal (i.p.) injections.

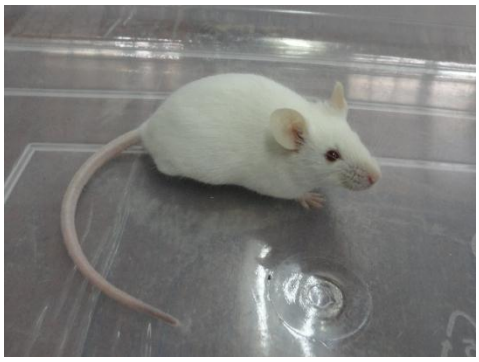


Figure 2-2: Normal Swiss Albino Mice

Figure 2-3: EAC Cell Bearing Swiss Albino Mice

Figure 2-4: Collection of intraperitoneal tumor cells

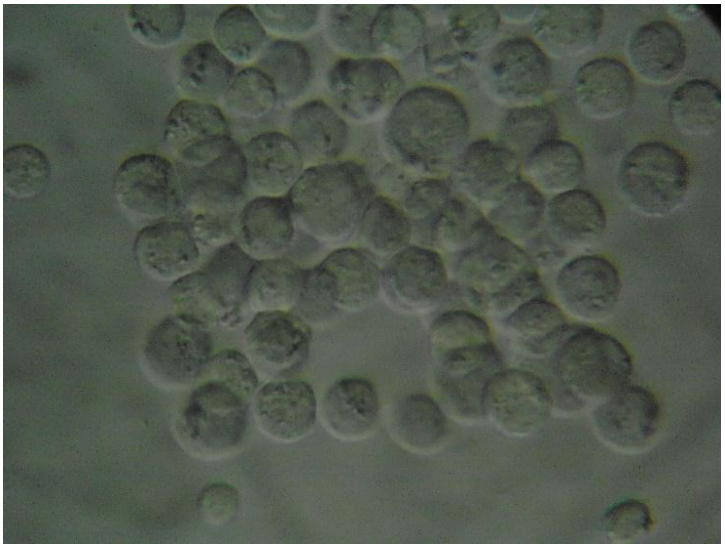


Figure 2-5: Ehrlich Ascites Carcinoma (EAC) Cells under microscope

2.2.3.4 Determination of EAC cell growth inhibition

a) Treatment with purified sword bean lectin (SBL): Ascitic fluid was drawn out from tumour bearing swiss albino mice. A 5 ml syringe fitted with 20 gauge needle was used for this tumor cell aspiration. The freshly drawn fluid was diluted with ice cool normal saline (0.98% NaCl solution) and the tumor cells number was adjusted to approximately 3.6 x 10^6 cells/ml by counting the cell number with the help of a heamocytometer. The viability of tumor cells was observed by trypen blue dye (0.4%) exclusion assay. Cell sample showing above 90% viability were used for transplantation. Tumor suspension of 0.1 ml was injected intraperitoneally (i.p.) to each swiss albino mice. Strict aseptic condition was maintained throughout the transplantation process. After 24 hours the mice were randomly distributed into five groups with at least 6 mice per group. Two groups received the test compound sword bean lectin (SBL) at different doses of (4mg/kg/day and 2.5 mg/kg/day) intraperitonealy respectively. One group was kept as positive control (EAC cell bearer) and another group received Bleomycin (0.3 mg/kg/day). Group 5 was kept as negative control. Treatment continued for 5 days.

On the 6^{th} day, mice in each group were sacrificed. The total intraperitoneal tumor cells were harvested by normal saline and coutned by a hemocytometer. The total number of viable cells in each mouse of the treated groups were compared with those of controls. Using the following formula, percent of inhibition was calculated:

Percent of inhibition = 100- {(cells from SBL treated mice/cells from control mice) X 100}

B) Treatment with carbonate apatite combined with SBL (*in vivo*): This experiment was done after inoculation of EAC cell in swiss albino mice. Among four groups of mice each containing 6 mice, two groups were treated with 5 mM carbonate apatite combined with sword bean lectin at the doses [4 ml/kg/day + 2.5mg/kg/day] and [4 ml/kg/day + 4mg/kg/day] respectively intraperitonealy. The remaining two groups were kept as positive and negative control.

Carbonate apatite was prepared according to Chowdhury E.H *et al.* 2007. SBL is mixed in a bicarbonate buffered medium containing necessary concentration of phosphate and calcium salts ($CaCl_2$). The mixture was incubated at 37°C for 30 min. Then microscopically visible carbonate apatite particles with major components of carbonate, phosphate and Ca^{2+} are formed (Chowdhury E.H. *et al.* 2006 and Chowdhury E.H. & Akaike 2007). Then treatment was

continued for 5 days. The total intraperitoneal tumor cells were harvested by normal saline and counted by a hemocytometer. The total number of viable cells in each mouse of the treated groups were compared with those of controls. Using the same formula, percent of inhibition was calculated:

Percent of inhibition = 100- {(cells from SBL treated mice/cells from control mice) X 100}

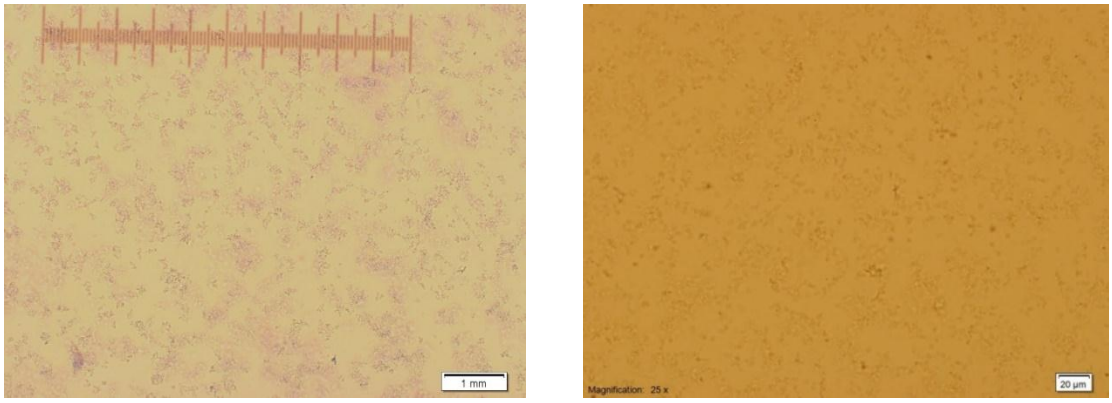


Figure 2-6: Microscopic Picture of 5 mM carbonate apatite

C) Treatment with Carbonate apatite (*in vivo*): Carbonate apatite was prepared as described in 2.2.3.4 (B)

After 24 hrours of inoculation of transplantable EAC cell in swiss albino mice according to the above mentioned process, the mice were randomly distributed into 3 groups containing at least 6 per group. One group was treated with 5 mM carbonate apatite {4ml/kg/day (i.p)}. One group was kept as positive control which is EAC cell bearer and the last group was kept as negative control. Treatment was continued for 5 days. The total intraperitoneal tumor cells were harvested by normal saline and counted by a hemocytometer. The total number of viable cells in each mouse of the treated groups were compared with those of controls. Using the following formula, percent of inhibition was calculated:

Percent of inhibition = 100- {(cells from SBL treated mice/cells from control mice) X 100}

2.2.3.5 Determination of Hematological Parameters of EAC cell Inoculated Mice with SBL, Carbonate Apatite and SBL Combined Carbonate apatite treatment:

The effect of above mentioned test compounds at the same dose on hematological parameters were studied in EAC cells inoculated mice by the method as described in Abbott, 1976 and Gupta *et al*. 2000. Treatment was started after 24 hours of EAC cell transplantation and continued for 10 days. Blood was drawn from the tail of each mouse on day 11. The percentage of hemoglobin measured by a hematometer and the total RBC were counted by using light microscope.

2.3 Results And Discussion:

2.3.1 Haemagglutination studies of purified sword bean lectin:

Hemagglutination assay has been widely used for the measurement of lectin activity. In the hemagglutination test, the microtritration format was employed in U-bottom poly styrene microtiter plate. In figure 2-7, row A indicates crude extract, row B indicates flow through, row C indicates affinity purified protein and row D indicates control *i.g.* only erythrocytes. Hemagglutination assay was performed using two fold serial dilution of sample against Tris-HCl buffer pH 7.5 with chicken erythrocytes. The chicken blood contains different sugar moieties on cell surface. Agglutination occurs when the lectin interacts with these sugar moieties. The RBC in the control wells have settled. The positive end-point was taken as the dilution that showed complete hemagglutination.

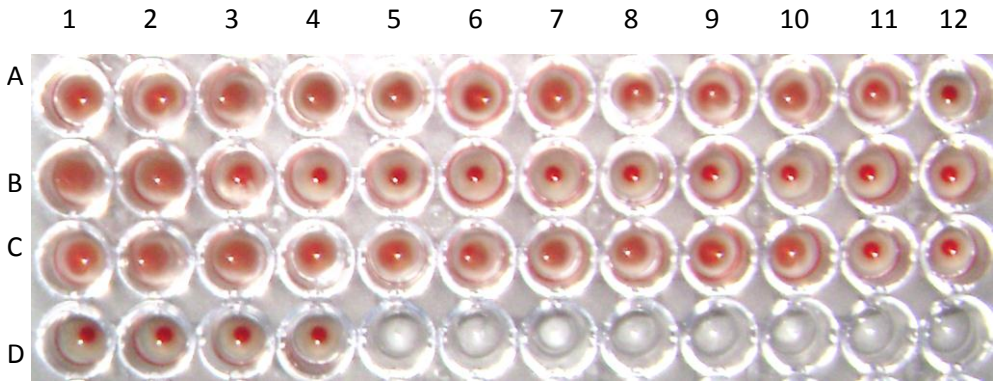


Figure 2-7: Photograph of hemagglutination tests in a microtiter plate. A: Crude extract, B: Flow through, C: Purified protein and D: Control.

Table 2-3: Hemagglutination activity of lectin. A: Crude extract, B: Flow through, C: Purified protein and D: Control.

	1	2	3	4	5	6	7	8	9	10	11	12
	Dilution (Sample: Buffer)											
	2	4	8	16	32	64	128	256	512	1024	2048	4096
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A	+	+	+	+	+	+	+	+	+	+	+	-
B	+	+	+	-	-	-	-	-	-	-	-	-
C	+	+	+	+	+	+	+	+	+	+	-	-
D	-	-	-	-								

Table 2-4: A summary of hemagglutination assay during affinity purification of lectin from sword bean.

Purification step	Volume (ml)	Lectin activity (Titer/ml)	Total activity (Titer)	Protein content (mg/ml)	Total protein (mg)	Specific activity (Titer/mg)	Fold purification	Yield %
Crude extract	6	2048	12288	27.9	167.4	73.41	1	100.00
Flow through	32	8	256	4.1	131.2	1.95	0.026	78.38
Affinity purified protein	11	1024	11264	2.15	23.65	476.3	**6.489**	14.13

From the hemagglutination assay the purification fold for affinity purified protein was found to be 6.489.

Hemagglutination assay was performed using chicken, mice, bovine, goat and human A, B, O groups' erythrocytes. Purified sword bean lectin powerfully agglutinated with mice erythrocytes, the order of hemagglutinating activity is mice> chicken> human blood A,B and O groups> bovine. It showed an equal agglutination activity with various human blood but not with bovine and goat erythrocytes. The minimum purified SBL concentration was found to be 5 µg/ml in chicken, 10 µg/ml in human blood types A,B,O and 0.0388 µg/ml in mice erythrocytes. Lectins were detected and quantified by their ability to agglutinate erythrocytes. The RBC contains different sugar moieties on the surface of the cell. Trypsination of blood increased the agglutination of cells compared to untrypsinized erythrocytes. Trypsin probably redistributes the receptor sites in the erythrocytes surface, which induces clustering that is more favorable for agglutination. The purified protein is a complete lectin since it agglutinates erythrocytes in saline solution without the aid of trypsin or proteolytic enzymes. The result of hemagglutination activity was presented in Table 2-5.

Table 2-5: Hemagglutination activity of lectin with various erythrocytes

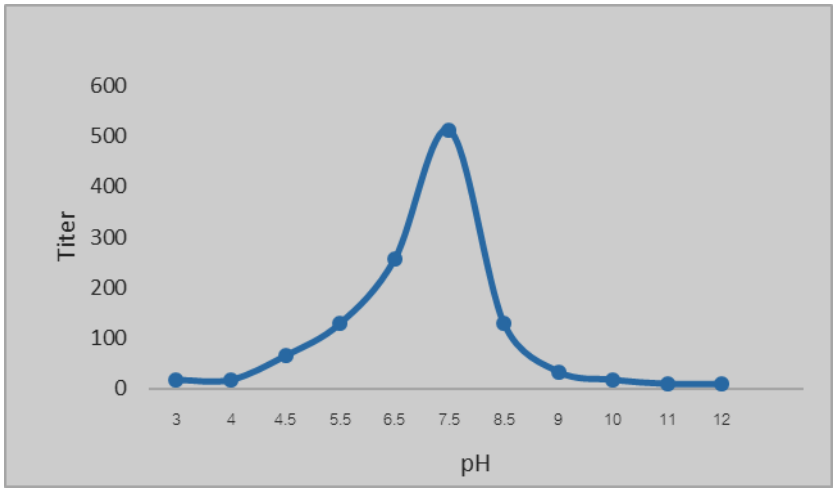
Lectin vs Erythrocytes	1	2	3	4	5	6	7	8	9	10	11
	Dilution (Sample: Buffer)										
	2	4	8	16	32	64	128	256	512	1024	2048
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Chicken	+	+	+	+	+	+	+	+	+	-	-
S. A. Mice	+	+	+	+	+	+	+	+	+	+	+
After 11	+	+	+	+	+	-	-	-	-	-	-
Bovine	-	-	-	-	-	-	-	-	-	-	-
Goat	-	-	-	-	-	-	-	-	-	-	-
Human O+	+	+	+	+	+	+	+	+	-	-	-
Human O-	+	+	+	+	+	+	+	+	-	-	-
Human A+	+	+	+	+	+	+	+	+	-	-	-
Human A-	+	+	+	+	+	+	+	+	-	-	-
Human B+	+	+	+	+	+	+	+	+	-	-	-
Human B-	+	+	+	+	+	+	+	+	-	-	-
Control	-	-	-	-	-	-	-	-	-	-	-

+ Hemagglutination activity, - No hemagglutination activity

2.3.2 Effect of pH and temperature on hemagglutination activity

The purified *Canavalia gladiata* lectin or SBL is stable upto 60°C after thermal denaturation and has a consistent titer value of 512. The titer value decreased at 80°C, it is devoid of lectin activity. This may be brought about by the denaturation of the lectin that removes its agglutination activity. The purified sword bean lectin showed highest hemagglutination activity at pH 7.5 and there was a gradual declination in lectin activity both above and below this pH. It is possible that changes in the ionization state with an increase in pH may lead to weaker binding of the metal ions, which are apparently required for maintenance of the structure that is required for maximal activity. The pH dependence, which is observed in virtually in all enzyme reactions, is a consequence of the protein composition. Numerous ionizable groups at the surface of the protein molecule and the active center are capable of reacting with H^+ or OH^-. Any pH changes are therefore associated with a change in the ionization state of the molecule, which in turn, determines the binding forces between enzyme and substrate (Adolph *et al*., 1982). It is also possible that the increase in OH^- ions caused a change in the ionization state of the lectin, thereby affecting the binding forces between the lectin and the erythrocyte membrane that eventually led to a loss of activity. In the following figure 2-8 the effect of temperature and pH on hemagglutination activity of sword bean lectin is shown.

A



B

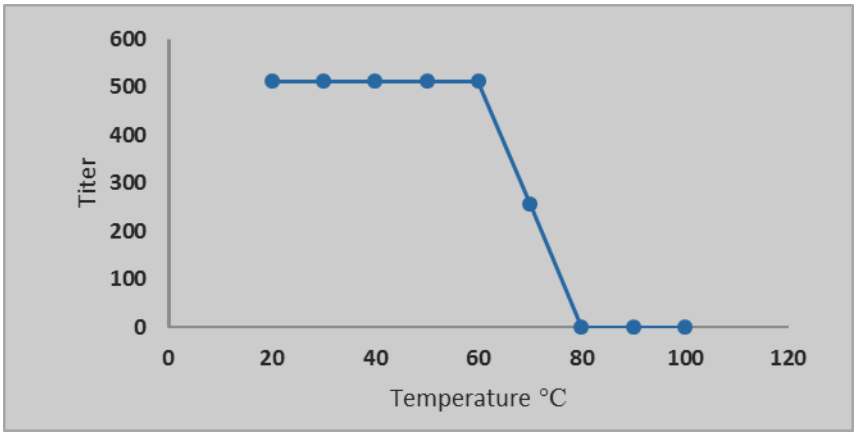


Figure: 2-8 Effect of (A) pH and (B) temperature on hemagglutination activity of purified sword bean lectin

2.3.3 Hemagglutination Inhibition Studies

RBC cell membrane possesses glycoproteins, sugars where the lectins can bind and cross bridge with each other. This agglutination may occur due to lectin sugar specificities or sugar binding sites in their structure. These sugar specificities of lectins can be measured most generally by the Landsteiner hapten-inhibition technique. (Landsteiner 1962).This is done by comparing sugars on the basis of the minimal concentration required to inhibit. The hemagglutination

inhibition of the purified SBL using chicken red blood cells was performed in the presence of different sugars and the result of hemagglutinin inhibition was presented in Table 2-8.

Table 2-6: Hemagglutination Inhibition study of sword bean lectin by mono and oligosaccharides against chicken erythrocytes:

Test Sugars	Concentration of sugars in mmol/l								
	50	25	12.5	6.25	3.125	1.5625	0.78123	0.3906	Ctrl
D(+) Glucose	-	-	+	+	+	+	+	+	+
Maltose	-	-	-	-	-	+	+	+	+
D(-) Mannose	-	-	-	-	-	+	+	+	+
D (+) Galactose	+	+	+	+	+	+	+	+	+
L(+) Arabinose	+	+	+	+	+	+	+	+	+
D(-) Arabinose	+	+	+	+	+	+	+	+	+
Fructose	-	-	-	-	-	+	+	+	+

We can see from table 2-8 that, sword bean lectin had specificity against D(+) glucose at Minimal Inhibition Concentration (MIC) of 25 mM, maltose, D(-) mannose, fructose, at MIC of 3.125 mM which inhibit hemagglutination activity. But lectins were not inhibited by the presence of D(+) galactose, L(+) arabinose.

2.3.4 Determination of EAC cell growth inhibition:

The antiproliferation activities of purified sword bean lectin and carbonate apatite was studied against Ehrlich Ascites Carcinoma (EAC) cells in swiss albino mice and the results were compared with Bleomycin, an anticancer drug. The results obtained are described below.

a) Treatment with purified sword bean lectin (SBL): Proliferation of EAC cells was effectively inhibited by SBL. When the dose of SBL was 2.5 mg/kg/day, the inhibition of the EAC cell growth was 45%, but when the concentration was raised to 4 mg/kg/day the growth

inhibition increased to 77% (Fig 2-9A). The EAC cell growth inhibition was found 90% when treated with Bleomycin, a well known anti-cancer drug. Hematological parameters were found to be different among normal, tumor bearing and SBL–treated tumor bearing mice. In figure 2-9(B) we can see the total RBC of the EAC cell bearing mice was 5.2 x 10^9. When the EAC cell bearing mice were treated with SBL (4 mg/kg/day), the RBC level increased to 8.6 x10^9 that is almost same with the level found in normal mice i.e. 9.4 x10^9. The hemoglobin level of tumor beaing mice also increased significantly after the treatment with SBL to 10gm/dl and the value was almost same to that of the normal mice which is 11.2 gm/dl (Fig 2-9C). Tumor growth was monitored for both SBL-treated and non-treated mice (Fig 2-9D) .

A

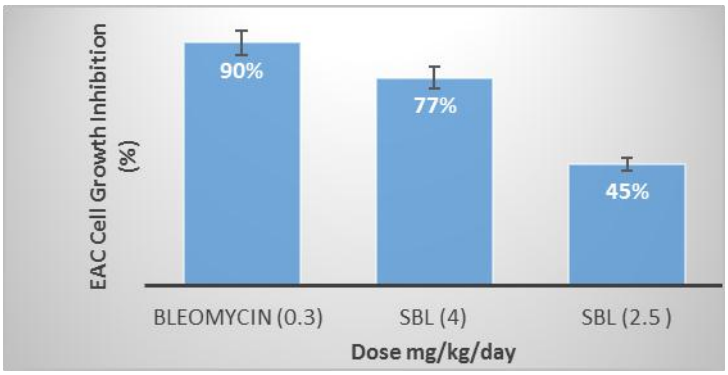


Figure 2-9(A): Effect of Bleomycin and SBL on the EAC cell growth inhibition *in vivo* in mice at 0.3 mg/kg/day for Bleomycin, 4 mg/kg/day and 2.5 mg/kg/day for SBL. Data are expressed in mean ±SD (n=6).

B

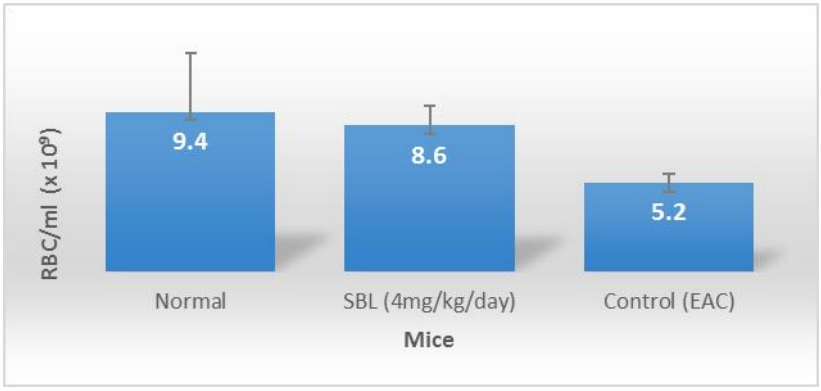


Fig 2-9(B): Effect of SBL at the dose of 4mg/kg/day on RBC *in vivo* in mice comparing with normal and EAC cell bearing mice**.** Data are expressed in mean ±SD (n=6).

C

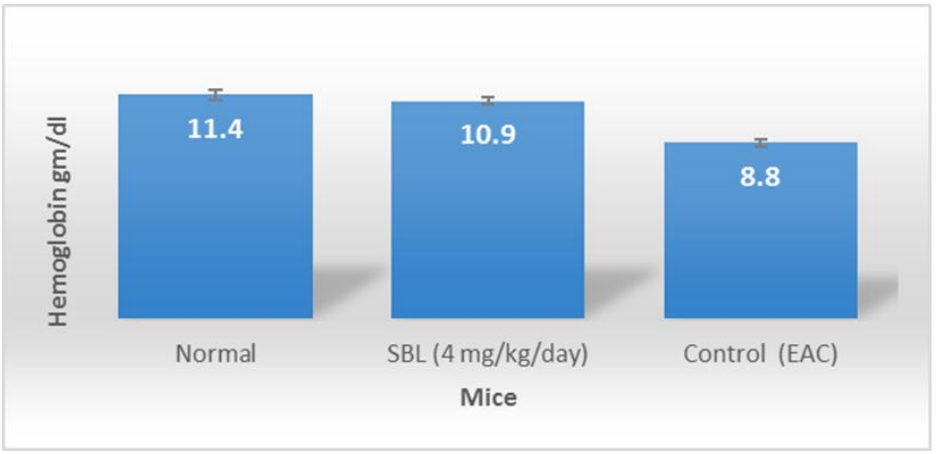


Fig 2-9 (C): Effect of SBL at the dose of 4mg/kg/day on Hemoglobin *in vivo* in mice comparing with normal and EAC cell bearing mice**.** Data are expressed in mean ±SD (n=6).

D

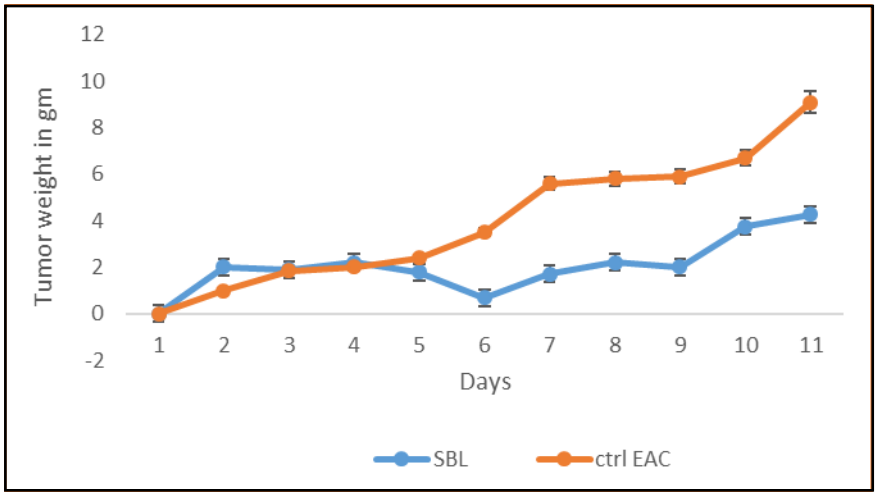


Fig 2-9 (D): Effect of SBL on average tumor weight growth comparing with EAC cell bearing mice**.** Data are expressed in mean ±SD (n=6). Blue line indicates the tumor weight growth of SBL treated mice and the orange line indicates the tumor weight growth of EAC cell bearing mice.

b) Treatment with Carbonate apatite combined with SBL (*in vivo*)

Literature shows that carbonate apatite can acts as drug carrier. To get a better antiproliferation rate of EAC cells carbonate apatite was used with lectin. So 5 mM carbonate apatite was used at 4ml/kg/day (i.p) combined with lectin and found that EAC cell growth inhibition decreases from 77% to 47% and 45% to 9% when 4mg/kg/day and 2.5mg/kg/day of SBL were used respectively (Fig 2-10). This was very much surprising. This result is indicating that carbonate apatite probably helps the EAC cell proliferation instead of inhibition. After observing this it was decided to treat the EAC cell bearing mice with carbonate apatite alone to see the actual effect.

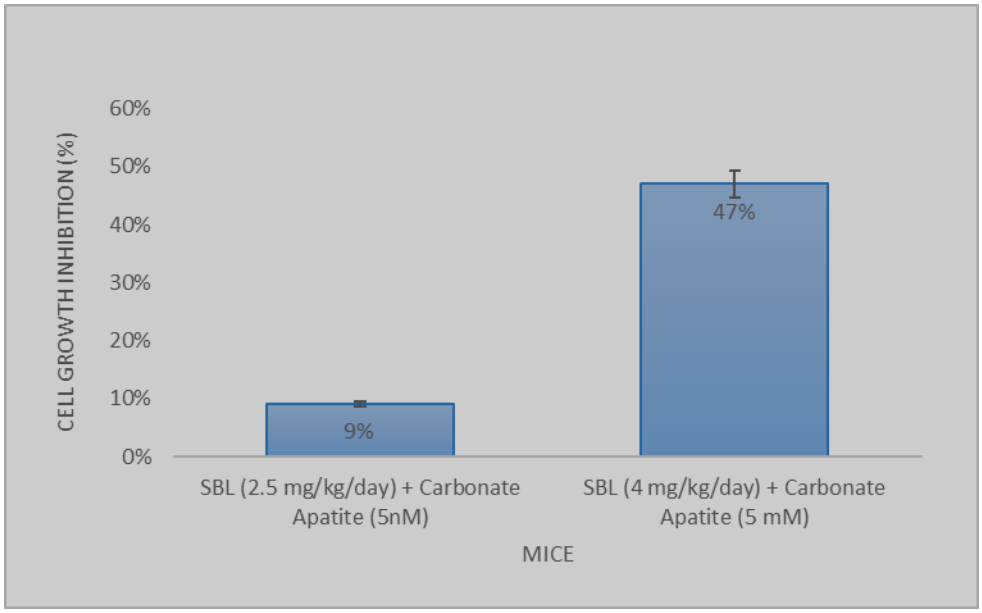


Figure 2-10: Effect of SBL (2.5 mg/kg/day) and SBL (4 mg/kg/day) combined with 5 mM carbonate apatite (4 ml/kg/day) on EAC cell growth in mice (*in vivo)*. Data are expressed in mean ±SD (n=6).

c) Treatment with Carbonate apatite (*in vivo*)

A

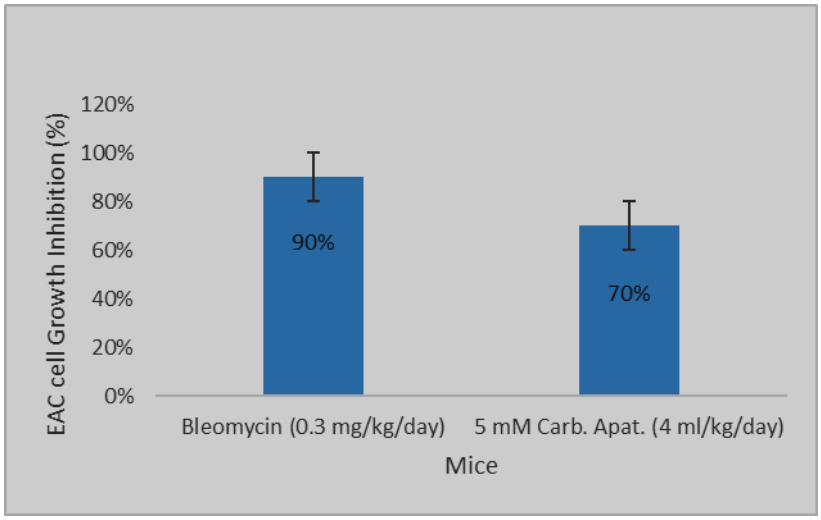


Fig 2-11 (A): Effect of Bleomycin (0.3 mg/kg/day) and 5mM carbonate apatite (4ml/kg/day) on EAC cell growth inhibition (*in vivo*) in mice. Data are expressed in mean ±SD (n=6).

B

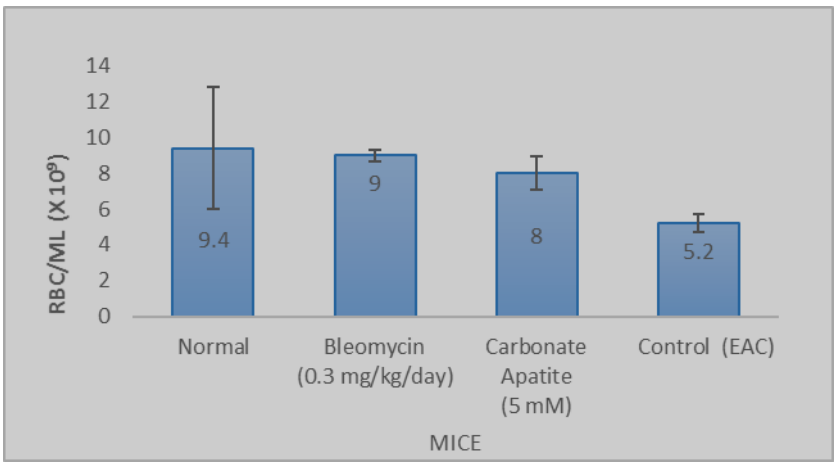


Fig 2-11 (B): Effect of Bleomycin (0.3 mg/kg/day) and 5mM carbonate apatite (4ml/kg/day) on RBC of EAC cell bearing mice (*in vivo*). Data are expressed in mean ±SD (n=6).

C

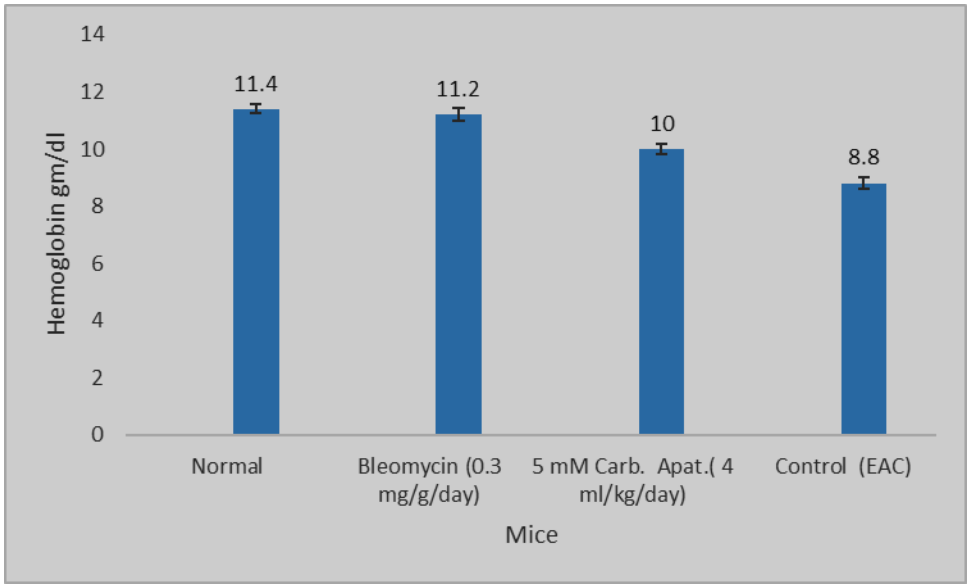


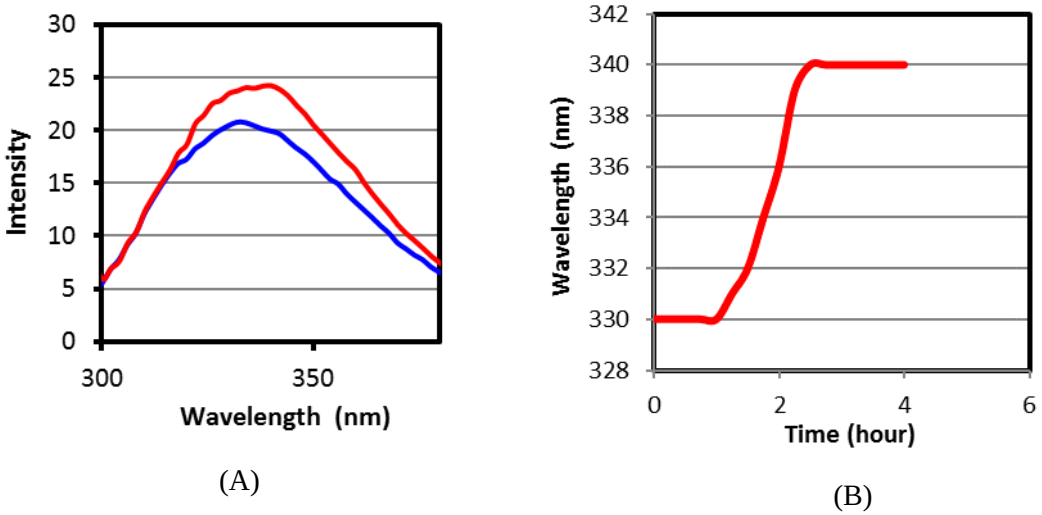
Fig 2-11 (C): Effect of Bleomycin (0.3 mg/kg/day) and 5mM carbonate apatite (4ml/kg/day) on Hemoglobin of EAC cell growth (*in vivo*) in mice. Data are expressed in mean ±SD (n=6).

It was quite surprising that carbonate apatite itself showed an interesting cell inbition rate which is 70% (Fig 2-11A). This result demanded to think about the combined effect of carbonate apatite and SBL. EAC cell growth inhibition using any apatite is not reported yet. RBC and haemoglobin of the carbonate apatite treated mice is found 8.0 and 10.9 respectively which is very close to normal value 9.4 and 11.4 (Fig 2-11B and C).

From the entire experiment it was observed that carbonate apatite and SBL individually showed significant EAC cell growth inhibition but when they are used combindly they are unable to inhibit EAC cell growth. This observation pushed to think about the surface induced denaturation of lectin.

In order to find out the reason fluorescence spectra of SBL was taken. SBL contains several tryptophan residues in molecular structure. Upon excitation at 280 nm, it gives emission at 330 nm. When the SBL become completely denature, there is a red shift to 340 nm. Blue line indicates the fluorescence emission spectrum of native SBL when excited at 280 nm and the red line indicates the fluorescence emission spectrum of SBL incubated with carbonate apatite for 1

hour (Fig 2-12A). The shift in λ_{max} from 330nm to 340nm clearly indicates that the SBL undergoes surface induced denaturation when incubated with carbonate apatite. Fig 2-12(B) indicates the surface induce denaturation profile of SBL in presence of carbonate apatite. X-axis indicates time in hour and Y-axix indicates wavelength in nm. It is clear from the figure that upto 1 hr the SBL remains in native state and after that it starts to denature. After 2 hr the SBL completely denatured as the λ_{max} value shifted to 340 nm.



(A) (B)

Figure 2-12: Effect of carbonate apatite on SBL observed by fluorescence spectroscopy**.** In fig 2-12(A) blue line indicates the fluorescence emission spectrum of native SBL when excited at 280 nm and the red line indicates the fluorescence emission spectrum of SBL incubated for 1 hour with carbonate-apatite. Fig 2-12B indicates surface induce denaturation profile of SBL in presence of carbonate apatite. X-axis indicates time in hour and Y-axix indicates wavelength λ_{max} in nm.

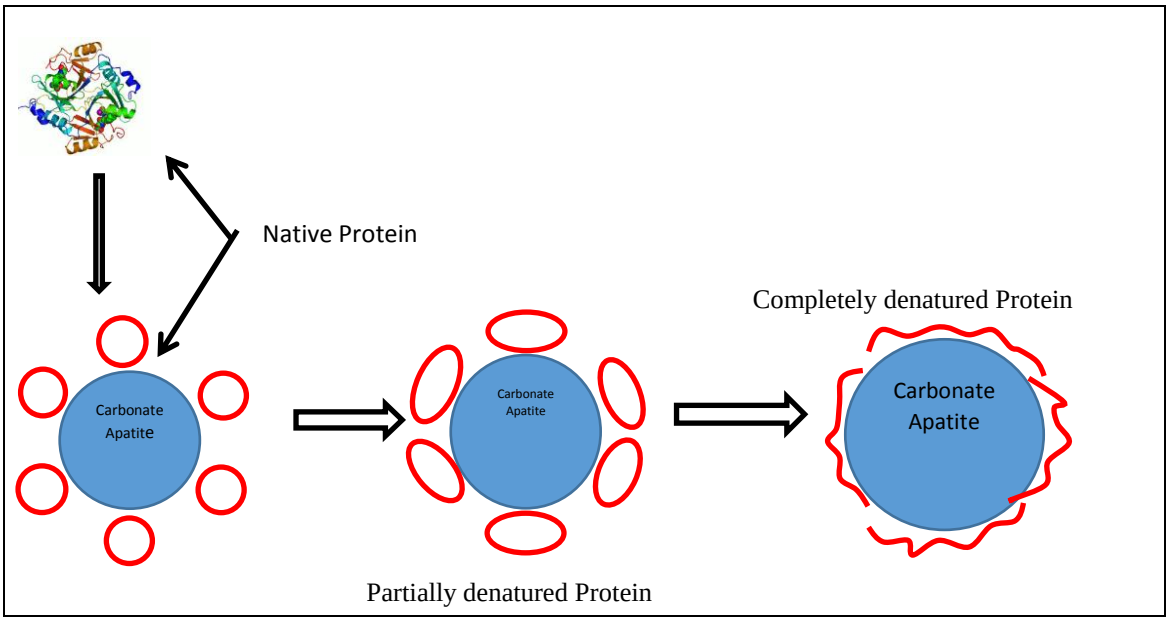


Figure 2-13: Schematic Representation of Surface Induced Denaturation Process

Figure 2-13 shows the possible schematic diagram of the unfolding procedure of lectin. At first, lectin molecule adsorb on the surface of carbonate apatite molecule. Then the lectin starts to unfold. At the last stage lectin completely denatured and cover the carbonate molecule by unfolding and thus both of them lose their activity.

2.4 Conclusion

The present study describes the hemagglutination and antiproliferative properties of a lectin purified from sword bean (*Canavalia gladiata*). The thermostability and pH stability of lectin (Ngai *et al.*, 2007) are known to differ from lectin to lectin. This investigation shows that SBL is considerably stable in the pH range 5-8 and maximum agglutinating activity at pH 7.5 whereas there was a gradual decline in lectin activity both below and above the pH value. The purified SBL was heat stable upto 60°C and has a consistent titer value of 512, beyond which the activity started declining. Similar observation has been reported for many lectins. Konozy *et al.*,(2003) reported about a D-galactose-binding lectin isolated from *Erythrina speciosa.* This lectin was acidic pH sensitive and remain active between pH 6.5 and 9.6. The EspecL was also stable below 65°C and became inactive when heated at 80°C for less than 10 min.

SBL powerfully agglutinated human blood types of A, B and O erythrocytes almost equally. In case of chicken and mice erythrocytes, hemagglutination activity is in the following order, mice >> chicken> human blood types A, B and O groups but not the bovine and goat erythrocytes. The hemagglutinating property of chicken erythrocytes with 1.0 μg/ml purified SBL has been inhibited 15-1106 μg/ml of different sugars (e.g. maltose, D- mannose and fructose).

There is evidence that plant lectins have shown unique characteristics against different types of cancer cells. Their effects involve death and growth inhibition of cancer cells. The two main properties of lecin; selectivity and cytotoxicity, have become the focus of attention in research against cancer. There is still the need to prove lectins for their possible use for the cancer treatment. Therfore, when the purified SBL was tested against Ehrlich Ascites Carcinoma (EAC) cell in the swiss albino mice at a dose of 4 mg/kg/day it worked as an active inhibitor. The efficiency of the compound has been compared with data obtained by running parallel experiments with a known effective anticancer drug, bleomycin at the dose of 0.3 mg/kg/day (i.p.). The cell growth inhibition was found to be 77% and 90% for SBL and bleomycin (Fig 2-9 A) when above doses were used respectively.

Tumor bearing mice became anemic due to decrease in RBC. When EAC cell bearing mice was treated with SBL at the same dose, the RBC level of EAC cell bearing mice increase from 5.2 to 8.6 (Fig 2-9 B) which is very close to normal value of 9.4. The hemoglobin level also increased from 8.8 to 10.0 (Fig 2-9 C) which is very near to normal level which is 11.4 gm/dl.

Cellular delivery involving the transfer of various drugs and bio-active molecules such as peptides, proteins and DNAs etc. through the cell membrane into cells has attracted increasing attention because of its importance in medicine and drug delivery. This led us to deliver SBL combining with inorganic carbonate apatite in different concentration to treat EAC cell bearing mice to get a better result. But this experiment gave us a decreased EAC cell growth inhibition from 77% to 47% and 45% to 9% at the dose of 4mg/kg/day and 2.5 mg/kg/day respectively (Fig 2-10) whereas 5 mM carbonate apatite (4ml/kg/day) itself gave a very good inhibition of 70% (Fig 2-11 A). The RBC and hemoglobin level after treating with carbonate apatite was 8.0 and 10 nearer to normal level of 9.4 and 11.4 respectively (Fig 2-11 B and C). Fluorescence study of SBL and SBL combined with carbonate apatite showed that surface induced denaturation (Fig 2-12) of SBL occurred when combined with carbonate apatite and prohibit the individual antiproliferation effect of sword bean lectin and carbonate apatite.

Chemicals

Important chemicals used in this study and their manufacturer are given below-

Acetic acid, Glacial

BDH, England

Acrylamide

Sisco Research Laboratories Pvt. Ltd., India

Ammonium sulfate, Analytical grade

BDH, England

Bromophenol blue

Fluka Chemika, USA

Calcium chloride, Laboratory grade

Merck, India

Coomassie brilliant blue R 250

Fluka Chemika, USA

D (+) dextrose

Fluka BioChemika, USA

Di-sodium hydrogen phosphate

Merck, India

Ethylene diammine tetra acetic acid disodium salt (EDTA)

BDH, England

Ethanol, 96%

BDH, England

Glycerine

Qualikems, India

Glycine

Bio-Rad Labratories, USA

Dextrose reagent

Crescent diagnostics, Saudi Arabia

Hydrochloric acid

Merck, India

Manganise chloride

BDH, England

2-Mercaptoethanol

BDH, Germany

Methanol

Reedel-dellaën, Germany

N, N′-methylene-bis-acrylamide

Loba Chemie, India

Sephadex G-200

Pharmacia Fine Chemicals, AB, Uppsala, Sweden

Sodium acetate

BDH, England

Sodium azide

BDH, England

Sodium chloride

Merck, Germany

Sodium dihydrogen orthophosphate

Loba Chemie, India

Sodium dodecyl sulphate (SDS)

Fluka Chemika, USA

Sodium hydroxide

Merck, India

TEMED

Sigma Chemicals Co., USA

Total protein kit

Human, Germany

Tris base

Loba Chemie, India

Trichloroacetic acid

Merck, Germany

Equipments

The important equipments used throughout this study and their model number origins are listed below-

HPLC (Gradient system with UV-Vis detector)

Jasco, PU-1580 (Pump), UV-1575 (Detector), Japan

T60 UV-Vis Spectrophotometer

PG instruments, England

Visible spectrophotometer

Erma AE-300, Japan

HiScale 26 column

GE Healthcare

Bio-mini UV monitor

AC-5200, Atto

Benchtop high speed centrifuge

Xiang Yi Centrifuge Instruments Co. Ltd., H-1880, China

Laboratory centrifuge

800, China

SDS-PAGE

Advantec, EP-150, Japan

pH meter

MP 220, England

Electric balance

Chyo, MP-3000, Japan

Refrigerator

Samsung, Korea

Hot Plate

Stuart Scientific, SH1, UK

0.5-10 µl micropipette

Nichipet EX Plus, Japan

1-10 µl micropipette

Huawei, China

1-20 µl micropipette

Gilson, France

10-200 µl micropipette

Gilson, France

10-100 µl micropipette

Huawei, China

1-100 µl micro pipettes

Huawei, USA

100-1000 µl micro pipettes

Kartell pluripet, P1001, Australia

100-1000 µl micro pipettes

Diapette, DI190, China

Magnetic stirrer

J- 6, USA

Magnetic stirrer with hot plate

Shanghai Huxi Analysis Instrument Factory Co. Ltd., 90-1, China

Vacuum pump

Speedivac ED150, England

Vortex mixture

Shanghai Huxi Analysis Instrument Factory Co. Ltd., WH-3, China

Dialysis bag

Diodesign Inc., D018, USA

Safety pipette filler

Deutsch & Neumann, Standard model, Germany

Filter paper

Double rings, 102, China

Dryer

RB, 500, Germany

Fluorescence spectrophotometer

Olympus IX71 Fluorescence microscope

Optical microscope

Common laboratory glass wares *i.e*. beaker, conical flask, pipette, funnel, test tube, measuring cylinder, volumetric flask, suction flask *etc*. were used.

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