2004

Analysis on the Nutrient compositions of Two Varieties of Sajna (Moringa Oleifera L.) Leaves and Purification, Characterization and Structure-function Analysis of Sajna Leaves Protein

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ANALYSIS ON THE NUTRIENT COMPOSITIONS OF TWO VARIETIES OF SAJNA (*MORINGA OLEIFERA* L.) LEAVES AND PURIFICATION, CHARACTERIZATION AND STRUCTURE-FUNCTION ANALYSIS OF SAJNA LEAVES PROTEIN

Thesis submitted to the University of Rajshahi, Bangladesh, in fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry and Molecular Biology.

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Session- July' 2000
August, 2004

CERTIFICATE

This is to certify that the materials included in this thesis are the original research works conducted by **Shahanaz Khatun.** The thesis contains no material previously published or written by another person except when due reference is made in the text of the thesis.

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DECLARATION

I do thereby declare that the material embodied in this thesis entitle **"ANALYSIS ON THE NUTRIENT COMPOSITIONS OF TWO VARIETIES OF SAJNA (*MORINGA OLEIFERA* L.) LEAVES AND PURIFICATION, CHARACTERIZATION AND STRUCTURE-FUNCTION ANALYSIS OF SAJNA LEAVES PROTEINS"** prepared for submission in the University of Rajshahi, Bangladesh for the degree of Doctor of Philosophy in Biochemistry and Molecular Biology are the original research works of mine and have not been previously submitted for the awards of any degree or Diploma anywhere.

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CONTENTS

ABSTRACT

Two types of Sajna leaves, small and large sized were analysed to obtained a comparative date on their chemical composition and nutritive values at different maturity levels.

The large sized leaves contained higher amount of TTA, dry matter, total chlorophyll, chlorophyll-a, chlorophyll-b, water soluble protein, lipid, total sugar, sucrose, starch, vitamin-C, vitamin-B_2 and iron while the small sized leaves contained higher amount of specific gravity, moisture, ash, total protein, total phenol, reducing sugar, β-carotene, vitamin-B_1, calcium and phosphorus. The nutrient compositions of Sajna leaves were found to be changed at different maturity levels. The specific gravity, dry matter, ash, lipid, total sugar, reducing sugar, sucrose, β-carotene, vitamin-B_2 and phosphorus content increased while TTA, moisture, starch, calcium and iron content decreased with the advancement of maturity in both type of leaves. The total chlorophyll, chlorophyll-a, chlorophyll-b, total protein, water soluble protein, total phenol, vitamin-B_1 and vitamin-C content were increased upto mature stage and then decreased in ripen stage.

The activities of protease and peroxidase enzymes increased with the changes of maturity, whereas amylase, cellulase, invertase, ascorbic acid oxidase and polyphenoloxidase increased upto mature stage and then decreased drastically in ripen stage. Fresh Sajna leaves contained significantly higher amount of oxidative enzymes as compared to the hydrolytic enzymes.

Polyphenoloxidase, a highly active enzyme in large sized Sajna leaves at mature stage was purified and characterized. The purification was accomplished by ion-exchange chromatography of crude enzyme extract on DEAE-cellulose followed by CM-cellulose chromatography. The purified enzyme gave single band on polyacrylamide gel indicating its homogeneity. The molecular weight of enzyme, as indicated by gel filtration and SDS-polyacrylamide gel electrophoresis were estimated to be 56,000 and 55,500 respectively and the enzyme contained only one subunit. The enzyme gave maximum activities in the pH range of 6.0-6.4 and at temperature 32°C. The

Km value of the enzyme was found to be 0.047 M using catechol as substrate. The purified PPO lost its activity completely in the presence of 4mM EDTA, while Ca^{2+} and Cu^{2+} at low concentration enhanced moderately the activities of the enzyme. The activity of PPO was abolished completely in presence of ascorbic acid, KCN and $NaHSO_3$.

Three lectins were extracted and purified from small sized Sajna leaves by Gel filtration of 100% ammonium sulfate saturated crude protein extract on Sephadex G-75 followed by ion-exchange chromatography on DEAE and affinity chromatography on Sepharose-4B. The lectins were found to be homogeneous as judged by polyacrylamide slab gel electrophoresis. The MW of the lectins SLL-1, SLL-2 and SLL-3 were 1,55,000, 1,15,000 and 85,000 respectively by gel filtration on sephadex G-150 and 1,60,000; 1,20,000 and 85,500 respectively by SDS-PAGE. SLL-1 and SLL-2 are dimer in nature which are held together by disulfide bond (s), while SLL-3 contain only one subunit i.e. it is monomer in nature. The lectins agglutinated specifically rat red blood cells and the agglutination was inhibited specifically by methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside and D-galactose. The lectins SLL-1 SLL-2 and SLL-3 contains 3.9, 3.4 and 2.8% neutral sugar respectively and the sugar composition of the lectins were glucose for SLL-1 and mannose for SLL-2 while SLL-3 contain either N-acetyl-D-glucosamine or N-acetyl-D-galactosamine or both. The lectins exhibited cytotoxic effect in brine shrimp lethality bioassay.

The biological activities of the lectins SLL-1 SLL-2 and SLL-3 were studied after various physical and chemical treatments. The biological activities of the lectins were affected greatly with the changes of pH and temperature and the lectins showed maximum hemagglutinating activities around pH 7.2-7.6 and temperature 20-35°C. The biological activities of the lectins were enhanced significantly in the presence of metallic salts Ca^{2+} while in the presence of EDTA, the activities of lectins were abolished completely.

LIST OF ABBREVIATION

DEAE-Cellulose	Diethylaminoethyl cellulose
BSA	Bovine serum albumin
CM- Cellulose	Carboxymethyl cellulose
PBS	Phosphate buffer saline
PPO	Polyphenol oxidase
PVP	Polyvinylpyrolidine
SLL-1	Sajna leaves lectin-1
SLL-2	Sajna leaves lectin-2
SLL-3	Sajna leaves lectin-3
MIC	Minimum inhibitory concentration
TLC	Thin Layer Chromatography

LIST OF TABLE

LIST OF FIGURE

General Introduction

GENERAL

Plants have great importance in human civilization. Human being and other living organisms depend on plant kingdom for many of their necessities of life. Plants supply food materials for men and animals. They provide human race with materials for clothing, shelter and fuel. Plants are unique in their ability to synthesize carbohydrates, proteins, fats and vitamins that constitute important nutrients of our diet. Plants also offer such substances as glycosides, alkaloids, antibiotics, sterols, saponins, essential oils, gum, resins, tannins etc. Plants also help us in maintaining air and environment.

Moringa (local name Sajna) is one of the most familiar plant of Bangladesh. It also have a medicinal value and is favoured to all the people of Bangladesh.

Sajna tree is generally found all over the country. The tree sheds it leaves from December-January. The new leaves appear at February-March. They are followed by flowers and whip-like tender fruits which ripen during summer.

Although, the tree is found all over Bangladesh, but it is found abundantly in Rajshahi, Rangpur, Dinajpur, Jessore, Kushtia, Bogra, Pabna and Chittagong.

ORIGIN

Moringa oleifera L. (Syn. *M perygosperma* Gaertn) is a plant of subtropical West Indies, Indian origin, widely distributed in the Indo-Bangla subcontinent and cultivated throughout the tropical belt (Sastri, 1962; Nadkarni,1976). A small genus of quick growing trees are distributed in Bangladesh, India, Arabia, Asia minor, Africa, Egypt and Australia. It is widely cultivated in tropics for its edible fruits.

SPECIES AND VARIETIES

It has been found that Sajna has only two species; of which one, *Moringa oleifera* Lam. is a familiar and small sized tree, leaves usually tri-pinnate,flowers white

while the other species, *Sajna concanensis* Nimmo is a small tree resembling *Moringa oleifera*, leaves bi-pinnate, somewhat longer than those of *Moringa oleifera*; flowers pinkish yellow. It is not available in our country. It is noted that both species are wild type. The size and shape of Sajna leaves are sometimes different from one plant to another. The size and shape of leaves are depended on the climatic conditions of growing area, soil texture etc.

SOIL AND CLIMATE

The Sajna tree requires warm weather and plenty of sunshine for its development. In the rainy season the presence of water in the soil causes the fully development of tree.

The tree is plentiful on recent alluvial land in or near sandy beds of rivers and streams. It is often cultivated in hedges and homeyards. It grows in all types of soils, except stiff clays and thrives best under the tropical insular climate. The tree can be propagated by seeds or from cutting; and cutting are preferred. Plants raised from seeds produce fruits of inferior quality. Further cutting of fairly large size, planted in moist soil, strike root readily and grow to sizeable tree within a few months. The stem is very soft. So, the major effects of unfavorable weather conditions are breakage of stems by storms and cyclones.

MEDICINAL IMPORTANCE

Different parts of this plant are used in the indigenous systems of medicine for the treatment of a variety of human ailments and are also eaten as vegetables (Chopra *et al.*, 1956; Sastri, 1962; Nadkarni, 1976; Ramchandran *et al.* 1980; Pushpangadan and Atal, 1968). The seeds are antipyratic, acrid and bitter. The seed oil is applied in rheumatism and gout (Chopra *et al.*, 1956 and 1958).The plant is also reported to have usefulness in case of gastrointestinal disorders such as stomach pain and dirrhoea (Dastur,1977).

Alkaloids: The root bark contains two alkaloids, viz. Moringine and Moringinine. The latter acts on sympathetic nerve endings and produces a rise in blood pressure, acceleration of heart beat and a constriction of blood vessels. Moringinine inhibits the tone and movements of involuntary muscles of gastrointestinal tract and relaxes bronchioles. An alkaloid, named spirochin, has been isolated from the roots; in high doses, which paralyses the vagus nerve (Chopra *et al.*, 1932-33;Chakravarti, 1957; Chatterjee and Mitra, 1951-52).

Antibiotics: Pressed juice of the leaves of the plant show strong antibacterial activity against *Micrococcus pyogens var.aureus*, *Escherichia coli* and *Bacillus subtilis*. The leaf juice is bacteriostatic in a dilution of 1:1000000 (Scharpenseel, 1956).

The roots contain an active antibiotic principle, pterygospermin which (in concentrations of 0.5-3μg/cc) inhibits the growth of many Gram-possitive and Gram-negative bacteria, including *Mycococuss pyogens var.aureus, Escherichia coli, Bacillus subtilis, Aerobacter aerogenes, Salmonella typhosa, Shigella desynteriae, S. enteritides, S. paratyphosus,* and *M. tuberculosis var. hominis*. In higher concentrations (7-10μg/cc) it is active against fungi. In view of its activity against fungi and mold, and negligible effect on seed germination, pterygospermin may find application in the preservation of fruits and vegetables and in seed treatment (Rao and Natarajan, 1949; Kurup and Rao, 1952; Rao and Kurup,1953; Kurup and Rao, 1954; Gopalakrishna, 1954).

GENERAL USES AND MEDICINAL IMPORTANCE

The tree is valued mainly for tender pods which are esteemed as vegetable. They are cut into slices and used in culinary preparations. They are also used as pickle. Flowers and tender pods are eaten as pod herb. Seeds are consumed after frying. They are reported to taste like peanuts. The roots of the tree are used as condiment

or garnish in the same way as those are pure horseradishes (*Cochlearia armoracia*). Twigs and leaves are lopper for fodder.

The leaves and pods contain vitamin A, vitamin C and are considered to be useful in scurvy and hay fever (Cacers *et al.*, 1991la; Gilani *et al.*; 1994). It also contains other vitamins. Vitamins are accessory food substances, acts as coenzyme of many enzymes, catalyzing and regulating biochemical reactions within the cell. Both the leaves and pods are rich in protein So, they meet the urgent need of our body and help to build our body.The pods contain histidine, arginine, methionine, threonine, valine, phenylalanine, tyrosine, leucine, isoleucine, tryptophan in trace amounts. The pods are remarkably rich in free leucine (Rau & Ranganathan, 1937; Kulkarni & Sohonie, 1956).

Gum: The stem of the tree exudes a gum which is locally used in calicoprinting (Howes, 1949; Ingle and Bhide, 1954).

Oil: The oils from the seeds of Sajna are now known in the trade as Ben or Behen oil, used locally for edible purposes, illumination, in cosmetics and as a lubricant for fine machinery.

The cake or meal left after extraction of oil from the kernels, has a bitter taste which is used as fertilizer

LECTINS

The term lectin was proposed by Boyd, (1970), refers a class of protein, which possesses the unique ability to agglutinate erythrocytes and other types of cells. Usually lectins are classified on the basis of sources i.e., plant lectin, vertebrate lectin, invertebrate lectin, lectin of protozoa, viral lectin, bacterial lectin etc.

Among them plant lectins are a heterogeneous group of carbohydrate- binding proteins comprising atleast seven distinct families of structurally and evolutionarily related proteins (Van Damme *et al.*, 1998). Four of these families, namely the legume lectins, the type-2 ribosome inactivating domains and the monocot mannose-binding lectins are considered to be 'large' families. The *amaranthins*, the *Cucurbitaceae*, phloem lectins and jacalin- related lectins comprise at present only a small number of individual lectins and accordingly are considered 'small' families. Amaranthins are T-antigen-specific lectins that have been found exclusively in a few *amaranthus* species. Similarly, the *Cucurbitaceae* phloem lectins are a small group of chitin- binding lectins confined to the phloem sap of a few genera of the family *Cucurbitaceae*. Jacalin related lectins which are named after T-antigen- specific lectins from jackfruit or *Artocarpus integrifolia*, occur in several species of the family *Moracea* and in a few unrelated species such as the jeru salem artichoke (*Helianthus turberosus*) and hedge bindweed (*Calystegia sepium*). In contrast with these 'smal' lectin families, the occurrence and distribution of the larger lectin groups has been studied in more detail. Legume lectins occur exclusively within the plant family *Leguminoseae*. Over 100 legume lectins have been characterized in detail (e.g.concanavalin A, phytohaemagglutinin etc.). Although all legume lectins are built up of protomers with high sequence similarities and strikingly similar three-dimensional structures but they differ from each other strongly with respect to their sugar-binding specificity. Type 2 RIPs have been found in plants from different families. Well-known examples of this family are ricin and abrin. In spite of their different taxonomico origins, all type of 2 RIPs consist of protomers with sequence similarities and very similar three- dimensional structure. Most type of 2 RIPs has similar though not identical specificities, usually directed against galactose or N-acetyl- galactosamine containing glycans. Chitin- binding lectins composed of hevein domains are widespread in the plant kingdom. Examples are wheat germ agglutinin and pokeweed mitogen. All members of this lectin family consist of protomers built up of one, two three, four or seven so- called hevein domains.

Both the amino acid sequences and three-dimensional structures of the hevein domains are markedly conserved, which explains why all chitin- binding lectins have similar carbohydrate binding specificities. In contrast with the other large lectin families, which have been studied intensively for several decades, the first monocot mannose- binding lectin was reported only in 1987 when a lectin with an exclusively specificity towards mannose was isolated from snowdrop (*Galanthus nialis*) bulbs (Van Damme *et al.*, 1987). Since then, related lectins have been found in various tissues of monocot families *Alliaceae, Amaryllidaceae, Araceae ,Bromeliaceae Iridaceae, Liliaceae* and *Orachidaceae* (Van Damme *et al.*, 1998). Biochemical analysis and molecular cloning clearly indicated that all these lectins belong to a single super family of mannose- binding proteins, which in accordance with their origin and specificity have been named monocot mannose-binding lectin (Van Damme *et al.*, 1995 and Barre *et al.*, 1996). At present, the monocot mannose- binding lectins are still being studied intensively because of their interesting biological properties, for example as potent inhibitors of retroviruses (Balzarini *et al.*, 1991 and 1992) and possible aplications in crop protection against insects and nematodes (Van Damme *et al.*, 1998).

Biological Roles

Lectins, the carbohydrate and cell agglutinating protein have found wide spread application as macromolecule probe for delineating the architecture and functioning of cell surface glyco-conjugates. Lectin is similar to enzyme in that they exhibit a specific interaction with particular molecule but by definition, restricted to interaction with sugar. Further lectins on cell surface mediate cell-cell interaction by combining with complimentary carbohydrates on opposing cells.

Role of Lectin in nature

Plant:

Attachment of nitrogen fixing to legumes.

Protection against phytopathogen.

Animals:

Endocytosis and intracellular translocation of glycoprotein.

Regulation of cell migration and adhesion.

Recognition determination in non immune phagocytosis.

Binding of bacteria to epithelial cells.

Microorganisms:

Attachment of bacteria and parasites (e.g. amoeba and plasmodium) to host cells.

Recognition determinants in non immune phagocytosis.

Recognition determinants in cell adhesion of slimemolds.

LITERATURE REVIEW

Tanzima Yeasmin *et. al.,(*2001) extracted and purified three lectins from mulberry seeds by gel filtration of 100% ammonium sulfate saturated crude protein extract followed by ion-exchange chromatography on DEAE and CM-cellulose. The lectins were homogenous and M_w of the lectins as determined by gel filtration were 175000 for MSL-1, 120,000 for MSL-2 and 89500 for MSL-3. MSL-1 is dimer in nature and the two monomers are held together by disulfide bond (s), while MSL-2 and MSL-3 contain four nonidentical subunits. The lectins agglutinated rat red blood cells and this agglutination was inhibited specifically by galactose, methyl-α-D galactopyranoside, methyl-β-D galactopyranoside, lactose and raffinose. The lectins exhibited strong cytotoxic effect in brine shrimp lethality bioassay.

M. Zoadur Rahman *et. al.*, (2001) investigated physico-chemical composition of healthy and diseased Sajna fruits at different maturity levels. Of varieties examined Najna contained highest amount of protein, total sugar, reducing sugar, total soluble solid (TSS), starch and ash while Sajna contained highest amount of total titratable acidity (TTA), moisture, lipid, vitamin C. In both the healthy and diseased conditions, total sugar, reducing sugar, sucrose, TSS, moisture, protein and lipid content were increased but ash, total titratable acidity (TTA), and vitamin C content decreased moderately with the changes of maturity. The activities of all the oxidative and hydrolytic enzymes studied were increased after infection of Sajna fruits with disease. The activities of catalase and protease increased but that of polyphenol oxidase and ascorbic acid oxidase decreased remarkably with the advancement of maturity stages and the activities of amylase, cellulase and invertase increased upto mature stage than decreased abruptly.

Mo *et al.*, (1999) purified the mannose/glucose-binding *Dolichos lablab* lectin from seeds of Dolicus lablab (hyacinth bean). The carbohydrate binding properties

of the purified lectin were investigated by hemagglutination inhibition assay, quantitative precipitation inhibition assay and ELISA.

Abo *et. al.*, (1999) estimated colorimetrically the anthraquinone content, antimicrobial and laxative effects of leaves and pods of *Cassia fistula* Linn., *C. specatabilis* DC and *L. podocarpa* because of the popular uses of these species. The pods of the *Cassia* species exhibits potent antifungal activity than the leaf samples. Pods of *Cassia fistula* showed significant antibacterial activity when compared to that of ampicillin. This study justifies the use of the *Cassia* species in traditional medicine.

Four sialic acid-specific lectins, ML-1, ML-2a, ML-2b and ML-3 were purified from leaves of the mulberry named Mon-Noi by ammonium sulfate precipitation, affinity chromatography on N-acetylgalactosamine agarose and gel filtration on Sephacryl S-200 (Ratanapo and Chulavatnatol, 1993). The hemagglutination inhibition study clearly suggested that four lectins were specific to N-glycolylneuraminic acid. The activities were also inhibited by high concentrations of N-acetylgalactosamine or galactose. On Sephacryl S-200, the native molecular weight of ML-1 was found to be higher than 669,000 and of ML-3 was found to be lower than 13,700. ML-2a and ML-2b were co-eluted on the Sephacryl S-200, having the native molecular weight of 44,000. The lectins ML-2a and ML-2b were found to be heat-labile glycoproteins containing 1.93% and 54.78% neurtal sugars, respectively.

Khomei Yanagi *et. al.*, (1990) extracted an anti-N lectin from *Vicia unijuga* leaves with phosphate buffered saline (PBS). Purification of the lectin was achieved, after pretreatment of the PBS extract by ammonium sulfate fractionation. Homogeneity of the purified lectin was demonstrated by HPLC and SDS-PAGE. The purified lectin was a glycoprotein with 11.4% carbohydrate and relatively high percentage of serine, threonine and aspartic acid residues and had a M_w of 120,000 Da. This

lectin agglutinated human N and MM erythrocytes but did not agglutinate M erythrocytes. Hemagglutination of the lectin was inhibited by glycophorin A^N and N- active sialoglycopeptide.

Lathyrus tingitanus seed lectins were purified by ammonium sulfate precipitation, affinity chromatography on Sephadex G-100 and subsequent chromatofocusing (Rough and Chabert, 1983). The amino acid composition, N-terminal amino acid, carbohydrate and metal content of both the lectins and their subunits were studied. This lectin was non-specific in agglutination of human erythrocytes and was inhibited by D- mannose, D- glucose and their α-methylglucosides derivatives.

A mitogenic lectin has been isolated and purified from *Lathyrus sativas* (Kolberg and Sletten, 1982). The lectin agglutinated human erythrocytes of different ABO groups and the agglutination is inhibited by D- mannose, D- glucose and their α-methylglucoside derivatives.

Franz *et al.*, (1981) isolated three lectins from an extract of mistletoe (*Viscum album*) by affinity chromatography. The lectins differ in molecular weight and sugar specificity. All three lectins react with human erythrocytes without specificity for the A, B and O blood groups. In contrast with abrin and ricin the mistletoe lectins could not be divided into toxin and hemagglutinins.

The hemagglutinating activity of *M. charantia* lectin was inhibited by galactose and other carbohydrates containing the galactopyranosyl residue (Mazumder *et al.*, 1981). From the chemical modification studies, tryptophan and tyrosine residue are found to be important for the carbohydrate binding activity of *Momordica charantia* lectin.

Vasi and Kalintha (1980) examined chemically the fruit pulp of *Cassia fistula* Linn. The fruit pulp of *Cassia fistula* is rich in proteins (19.94) and carbohydrate (26.30).

In the tomato (*lycopersicon esulentum*) plant, the fruit juice was found to be the richest source of agglutinating activity (Kilpatrick, 1979). The lectin responsible could be inhibited by oligomers of N-acetylglucosamine, and this property was exploited to purify the lectin by affinity adsorption on trypsin- treated erythrocytes. The lectin is a glycoprotein that cross-reacts immunologically with the lectin from *Datura stramonium* (thorn-apple).

The biological activities of Ricin D were investigated after physical and chemical treatments (Taira *et al.*, 1978). The results demonstrated that the intact Ricin D was stable in all the pH ranges but its activity destroyed drasticaly above 60°C. Chemical modification of Ricin D with specific reagents revealed that the tryptophan and tyrosine residues as well as carboxy groups participated in the phenomena of cytoagglutination and toxic action of Ricin D.

Gupta *et. al.*, (1975) carried out a research work on chemical composition and in vitro nutrient digestibility of some of the tree (including *C. fistula*) leaves. The dry matter of leaves of 23 different species of trees ranged (17.2-49.3%), crude protein (9.2-21.0%), neutral detergent fiber (18.5-76.0%), total mineral (9.25-21.5%), Ca (1.12-4.0%), P (0.01-0.06%) and SiO_2 (0.3-5.2%). Leaves of most species could be used for animal feed.

Paulova *et al.*, (1871a) reported that Mn^{+2} and Ca^{+2} enhances both the hemagglutinating and polysaccharide precipitating activities of phytohemagglutinins, isolated from the lentil. Further, they found that EDTA strongly inhibits mannan precipitation and to a smaller degree, erythrocyte agglutination.

Aim of the present study

A large number of population in our country has been suffering from malnutrition. There are many kind of vegetables available in our country, which are rich in nutrients. Sajna leaves are one of them. For the ignorance of people, they do not know the nutritive value of different kinds of vegetables.

The leaves contain considerable amount of vitamin-A and vitamin-C. It also contain other vitamins, which are accessory food substances, act as coenzymes of many enzymes, catalyzing and regulating biochemical reactions within the cell. Sajna leaves are rich in protein. So, they meet the urgent need of our body help to build our body. The leaf juices are reported to have antibacterial activity against a number of gram negative microorganisms like *E coli*, *Bacillus substilis* etc. as well as bacteriostatic activity.

Although Sajna are produced abundantly in our country but data available on the physico-chemical parameters of Sajna leaves produced in Bangladesh is very scantly. No detail work has so far been done on the physico-chemical properties and nutritive values of Sajna leaves of different varieties.

Plants contain lectins. Lectins are sugar-binding proteins that agglutinate cells and / or precipitate glycoconjugate molecules with a carbohydrate. Lectins were first called phytohemagglutinins since they were originally only isolated from plant extract and were used for the agglutination of blood cells.

Lectin plays a key role in the control of various normal and pathological process in living organisms. Research in the field of lectins has been going on in many research laboratories of the world. So far more than hundred lectins have been purified and characterized but their molecular mechanism has yet not been understood.

One of the advantages of studying lectins in that the characteristics of these proteins, as well as the oligosaccharides that bind to them, are well known. The preliminary research in our laboratory has established that the Sajna leaves contain more than one galactose specific lectins. Since lectins isolated from different sources have unique specificities and are useful reagents for glycoconjugates separation as well as possess many biological properties, so many laboratories in different country have been continuing research works in the field of lectins. Considering the need of novel lectins, we first time continued our research works on Sajna leaves lectins.

The thesis consists of:

a) Physico-chemical properties such as pH, specific gravity, total titratable acidity (TTA), moisture, dry matter, ash, chlorophyll, protein, lipid, total sugar, reducing sugar, sucrose, starch, β-carotene, riboflavin, thiamine, ascorbic acid, minerals such as calcium, iron and phosphorus contents in both small and large sized Sajna leaves were analyzed at different maturity levels.

b) Changes in enzyme contents such as amylase, protease, cellulase, invertase, ascorbic acid oxidase, polyphenol oxidase and peroxidase were determined at different maturity levels in both type of leaves.

c) Purification and characterization of polyphenol oxidase from large sized Sajna leaves at mature stage.

d) Purification and characterization of lectins from small sized Sajna leaves.

e) Effects of physico-chemical agents on the stability of lectins purified from Sajna leaves.

CHAPTER-1

An analysis of the physico-chemical compositions of two different types of Sajna leaves at different maturity levels.

1.1. INTRODUCTION

Sajna leaf is one of the most familiar and widely available vegetables of Bangladesh. It has an important role as a source of proteins, carbohydrates, vitamins and other nutrients in human diet, which are necessary for maintaining proper health.

Sajna leaves are not affected by any diseases, but there is a transformation in the leaves. Chlorophyll is responsible for the green color of leaves. The young green leaves are transformed into yellowish green when ages, which may be explained with the following explanation-

the young, green tomato ages and ripens and as its cells senesce, their chloroplast lose Chlorophyll and protein but synthesize carotenoids, which have a yellow to red color, depending on the tomato (Watson M. Laetsch, Basic concepts in botany).

The biochemical composition of Sajna leaves of different types grown in the climatic condition of Bangladesh are yet not known. Therefore, in present investigation, two types of Sajna leaves have been selected to analyze their physico - chemical parameters at different maturity levels.

1.2. MATERIALS AND METHODS

1.2.1. Materials

Two different types of Sajna leaves named "small sized leaves" and "large sized leaves" were collected during the stages of immature (light green), mature (deep green) and ripen (lighter or more yellowish green). These leaves were collected from the same varieties tree from Kazla, Rajshahi, Bangladesh. Day required from the time of leaf set were upto 10±4, 30±10 and 50±10 days for immature, mature and ripen stages respectively. Some information about these types are given below:

Small sized leaf: This is the most available variety in Bangladesh. The tree of this type of leaf is relatively high and more branched. These type of leaves are thick and very small in size. It ranges from 12±3 mm in length and 8±2 mm in width. As shown in Fig.1.1. the leaves became deep green at mature stage, lighter or more yellowish green color at ripen stage.

Large sized leaf: This type is not available very much in Bangladesh. The tree of this type is high and less branched. The leaves are thin and relatively large in size. It ranges from 30±5 mm in length and 20±4mm in width. Like small sized type the leaves of this type also became deep green at mature stage and lighter or more yellowish green at ripen stages (Fig.1.2.).

Fig. 1.1: Photograph showing "Small sized Sajna leaves" at mature and ripen stages.

Fig. 1.2: Photograph showing "Large sized Sajna leaves" at mature and ripen stages.

1.2.2. Methods

Freshly harvested Sajna leaves at different maturity levels were brought to the Protein and Enzyme Research Laboratory, Department of Biochemistry and Molecular Biology, Rajshahi University for experimental purposes. The physical and chemical parameters were studied by the following methods.

1.2.2.1. Determination of pH

Sajna leaves (2g) were crushed thoroughly in a mortar with a pestle and homogenized well with 30 ml of distilled water and then filtered through two layers of muslin cloth. The filtrate was then centrifuged for 10 min at 5000g and the clear supernatant was collected. The pH of the extracted juice was determined by a Corning 215 pH-meter using standard buffer solution.

1.2.2.2. Determination of total titratable acidity (TTA)

The juice of Sajna leaves was extracted by the procedure as described above and the total titratable acidity of the extracted juice was determined by Folin's method (Oser, B.L., 1965).

1.2.2.3. Determination of specific gravity

The specific gravity (Sp.gr.) of Sajna leaves juice was determined by means of a specific gravity bottle using the formula (M. Kalimuddin, 1976).

1.2.2.4. Determination of moisture content of Sajna leaves.

Moisture content of Sajna leaves was determined by weight loss of the sample on drying at 105°C for 5 hours (ICOMR,1971).

1.2.2.5. Determination of dry matter content of Sajna leaves.

Dry matter content was calculated from the data obtained for percentage of moisture content.

1.2.2.6. Determination of ash content of Sajna leaves.

Ash content was determined following the method of A.O.A.C.(1980).

1.2.2.7. Extraction and estimation of chlorophyll in Sajna leaves.

Chlorophyll is the green pigments universally present in all the photosynthetic tissues. Chlorophyll-a and chlorophyll-b occur in higher plants. The extraction of chlorophyll was carried out with 80% acetone from the leaves. After filtration, the filtrate was pooled and made upto 100ml in a volumetric flask with 80% acetone and the absorbance of this extract was measured at 645 nm and 663 nm for the determination of Chlorophyll-a and Chlorophyll-b respectively. The chlorophyll content of the small and large sized Sajna leaf tissues were calculated employing the formula using the specific absorption coefficient for Chlorophyll-a and chlorophyll-b at 645 nm and 663 nm in 80% acetone respectively as described in Methods of Physiological Plant Pathology (Mahadevan & Sridhar, 1982).

$$\text{Total chlorophyll (mg/g)} = \frac{20.2A_{645} + 8.002A}{1 \times 1000 \times w} \times v$$

$$\text{Chlorophyll–a (mg/g)} = \frac{12.7A_{663} - 2.69A_{645}}{1 \times 1000 \times w} \times v$$

$$\text{Chlorophyll–b (mg/g)} = \frac{22.9A_{645} - 4.68_{663}}{1 \times 1000 \times w} \times v$$

Where, A= Optical density in each cases

I= Length of light path in the cell (usually 1 cm).

V= Volume of the extract in ml and

W= Fresh weight of the sample in g.

1.2.2.8. Determination of total protein content of Sajna leaves.

Total protein content of different varieties of Sajna leaves was calculated from total nitrogen by using N × 6.25 after determination of the total nitrogen by the method of Micro-Kjeldahl (Jayaraman 1981).

1.2.2.9. Determination of water soluble protein content of Sajna leaves.

Water soluble protein content of Sajna leaves was determined by the Folin-lowry method (Lowry, 1951). The extraction was carried out with distilled water. The amount of water soluble protein present was calculated by constructing a standard curve with Bovine serum albumin.

1.2.2.10. Determination of total phenol content of Sajna leaves.

Total phenol content of Sajna leaves was determined colorimetrically by Folin-Ciocalteau's method (Bray and Thorpe, 1954). Extraction of phenol from Sajna leaves was done following the method as described by Loomis and Shull,(1937).The amount of total phenols was calculated from the standard curve of catechol.

1.2.2.11. Determination of lipid content of Sajna leaves.

Lipid content of different varieties of Sajna leaves was determined by the method of Bligh and Dyer (1959) using a solvent mixture of chloroform and ethanol (2:1 v/v).

1.2.2.12. Determination of total sugar content of Sajna leaves.

Total sugar content of Sajna leaves was determined colorimetrically by the anthrone method (Dubois *et. al.*, 1951).Extraction of sugar from Sajna leaves was done following the method of Loomis and Shull (1937) and the amount of total sugar present was calculated by constructing a standard curve with glucose.

1.2.2.13. Determination of reducing sugar content of Sajna leaves.

Reducing sugar content of Sajna leaves was determined by dinitrosalicylic acid method (Miller, 1972). Extraction of sugar from Sajna leaves was done following the method of Loomis and Shull (1937) and the amount of reducing sugar was calculated by constructing a standard curve with glucose.

1.2.2.14. Determination of sucrose content of Sajna leaves.

Sucrose content was calculated from the following formula (Ranganna,1979):

% Sucrose or non-reducing sugar = (%Total sugar- %reducing sugar) x 0.95

1.2.2.15. Determination of starch content of Sajna leaves.

The starch content of Sajna leaf was determined by the anthrone method as described in Laboratory method in Bio-chemistry (Jayaraman, 1981).

A standard curve of glucose was prepared and the amount of starch in the Sajna leaf was calculated from the graph.

1.2.2.16. Determination of β-carotene (as Vitamin-A) Content of Sajna leaves.

β-Carotene content of Sajna leaves was determined according to the procedure described by Jensen (1978). The amount of β-carotene was calculated by constructing a standard curve with standard β-carotene.

1.2.2.17. Determination of B-vitamins content of Sajna leaves.

Vitamin-B_1 (Thiamin):

Vitamin-B_1 content of Sajna leaves was determined following the method of Anon(1965).

Vitamin-B_2 (riboflavin):

Vitamin-B_2 content of Sajnae leaves was determined by the method of Anon (1965).

1.2.2.18. Determination of Vitamin-C content of Sajna leaves.

Vitamin-C content of Sajna leaves was determined by the titrimetric method (Bessey and King, 1933).

1.2.2.19. Determination of mineral content of Sajna leaves.

A) **Calcium :** Calcium content of the Sajna leaves was determined by the method as described in Practical Physiological Chemistry (Basset, J. 1978). The amount of calcium was calculated by constructing a standard curve with calcium carbonate solution.

B) Iron: Iron content of Sajna leaves was determined following the method as described by Ranganna (1986).

The amount of iron was calculated by constructing a standard curve with ammonium iron (111) sulfate solution.

C) Phosphorus : Phosphorus content of Sajna leaves was determined by the method of Ranganna (1986). The amount of phosphorus was calculated by constructing a standard curve with potassium dihydrogen phosphate solution.

1.3. RESULTS AND DISCUSSION

1.3.1. pH and TTA of Sajna leaves

The pH and TTA of Sajna leaves of the small sized and large sized Sajna leaves at different maturity levels are given in Table –1.1.

The pH of Sajna leaf is in the acidic range at all the maturity levels. From the result of pH and TTA, it may be concluded that the acidity of Sajna leaf decreased gradually with the advancement of maturity. Of the two types analysed the small sized leaf is more acidic than the large sized leaf. Further pH value of 5.95 and above is an indication of ripening of Sajna leaf. The decrease in acidity with the advancement of maturity levels may be due to more metabolic activities at these stages.

1.3.2. Specific Gravity

Specific Gravity of Sajna leaf's juice describes how many times its weight is more than water.

Data presented in Table-1.1 showed that Specific Gravity of small sized Sajna leaf was 0.65 in immature stage, 0.88 in mature stage and 1.07 in ripen stage, while that of large sized leaf was 0.60 in immature stage, 0.74 in mature stage and 0.99 in ripen stage.

The results clearly indicated that the Specific Gravity of Sajna leaf increased slightly with the changes of maturity. Further the Specific Gravity of small sized leaf was higher as compared to that of the large sized leaf at all the maturity stages. The similar result was reported by Karim *et al.*, (1996) who stated that Specific Gravity of tomato flesh increased with the advancement of maturity. The increased in Specific Gravity with the advancement of maturity might be due to increased concentration of soluble materials.

Table-1.1: pH, TTA and Specific gravity of Sajna leaves at different maturity levels. (on the basis of fresh weight).

	Types of leaves	Stages of Maturation		
		Immature	Mature	Ripen
pH	Small sized leaves	4.00±0.05	5.30±0.06	5.95±0.05
	Large sized leaves	4.35±0.04	5.50±0.06	6.02±0.03
TTA (ml of 0.1N NaOH required/100g)	Small sized leaves	25.2±0.02	19.0±0.02	17.2±0.01
	Large sized leaves	31±0.05	22±0.07	18.5±0.06
Specific gravity	Small sized leaves	0.65±0.002	0.88±0.004	1.07±0.002
	Large sized leaves	0.60±0.004	0.74±0.003	0.99±0.005

1.3.3. Moisture content of Sajna leaves

Moisture plays an important role in the growth activities of trees as well as is essential for most of the physiological reactions in the plant tissues. Moisture content of Sajna leaf of two types are presented in Table-1.2. The moisture content were found to be varied between 75.9-83.3% and 67.06-75.01% in small sized and large sized Sajna leaves at different maturity stages respectively. The results also revealed that the moisture content of Sajna leaf decreased gradually with the advancement of maturity levels in both types of leaves. The decreased in moisture content might be due to accumulation of solid material in the leaves with the changes of maturity.

1.3.4. Dry matter content of Sajna leaves

Data on changes in dry matter content of Sajna leaves derived from percentage of moisture content are shown in Table-1.2.

The results indicated that the dry matter content of Sajna leaves increased remarkably in both types with the advancement of maturity. The increased in dry

matter content shows good correlation with the decrease in moisture content. Similar results were also reported for mango leaves by Jahangir Kabir(1997).

1.3.5. Ash content of Sajna leaves

The ash content of Sajna leaves are shown in Table-1.2.

The present data indicates that the ash content of Sajna leaves were increased remarkably with the changes of maturity in both types of leaves. The increase in ash content might be due to increased amount of solid materials.

Ferdaus and Haque (1989) also reported that the ash content of banana increased gradually with the changes of maturity. The ash content obtained in the present study was comparable with that of Sajna leaves produced in India (Sastri, 1962).

Table-1.2: Moisture, dry matter and ash contents of Sajna leaves at different maturity levels.

	Types of leaves	Stages of Maturation		
		Immature	Mature	Ripen
Moisture(%)	Small sized leaves	83.3±0.02	82.5±0.03	75.9±0.01
	Large sized leaves	75.01±0.04	72.04±0.05	65.06±0.09
Dry matter(%)	Small sized leaves	16.7±0.01	17.5±0.03	24.1±0.04
	Large sized leaves	24.99±0.03	27.96±0.02	32.94±0.01
Ash(%)	Small sized leaves	2.47±0.001	3.78±0.0011	5.28±0.002
	Large sized leaves	2.0±0.002	3.25±0.003	4.8±0.0021

1.3.6. Chlorophyll content of Sajna leaves

Total chlorophyll, chlorophyll-a and chlorophyll-b contents of two types of Sajna leaves are presented in Table-1.3. Large sized leaves contain highest amount of all types of chlorophyll in the mature stage (Total chlorophyll-2.58 mg g^{-1} chlorophyll-a, 1.58 mg g^{-1} and chlorophyll-b 0.993 mg g^{-1} of leaf) than small

sized leaves (Total chlorophyll, 1.66 mg g^{-1}; chlorophyll-a,1.06 mg g^{-1} and chlorophyll-b 0.599 mg g^{-1} of leaf).

The chlorophyll content of Sajna leaves was increased upto mature stage and thereafter i.e. in ripen stage its content was decreased drastically. This result also supported from the physical observation that the color of leaves become light yellowish green in ripen stages.

In ripen stages, the large sized leaves contain lowest amount of all types of chlorophyll (Total chlorophyll-0.32 mg g^{-1}; chlorophyll-a, 0.171 mg g^{-1} and chlorophyll-b 0.126 mg g^{-1} of leaf) than small sized leaves (Total chlorophyll-0.35 mg g^{-1}; chlorophyll-a, 0.199 mg g^{-1} and chlorophyll-b 0.135 mg g^{-1} of leaf) Watson M. Laetsch ("Plants Basic concepts in Botany"-Book) reported that, during ripening, the chloroplast of leaves lost chlorophyll and synthesized carotenoids; and this trend may be in line with the present findings.

Table-1.3: Total Chlorophyll, Chlorophyll-a and Chlorophyll-b contents of Sajna leaves at different maturity levels.

	Types of leaves	Stages of Maturation		
		Immature	Mature	Ripen
Total Chlorophyll (mg g^{-1})	Small sized leaves	0.728±0.01	1.66±0.02	0.35±0.03
	Large sized leaves	1.69±0.02	2.58±0.01	0.32±0.01
Chlorophyll-a (mg g^{-1})	Small sized leaves	0.633±0.01	1.06±0.02	0.199±0.01
	Large sized leaves	0.68±0.08	1.58±0.07	0.171±0.06
Chlorophyll-b (mg g^{-1})	Small sized leaves	0.214±0.02	0.599±0.01	0.135±0.01
	Large sized leaves	0.65±0.02	0.993±0.01	0.126±0.03

1.3.7. Total protein and water soluble protein content of Sajna leaves.

The protein constituents of fruits and vegetables, although occurring in low concentration, are of primary importance not only as component of nuclear and cytoplasmic structures, but also including, as they must the full complement of enzymes involved in metabolism during growth, development, maturation of fruit and vegetables (Hansen, 1970).

As shown in Table 1.4, the small sized leaves contained 5.88%, 7.65% and 4.1% while the large sized leaves contained 5.58%, 7.2% and 4.0% of total protein in immature, mature and ripen stages respectively.

The results clearly indicated that protein content of Sajna leaves increased until the end of the leaf growth and then decreased at the ripen stage. The decrease in protein from the mature to ripen stage might be due to the hydrolysis of protein by enzymes.

From the present result it can be suggested that Sajna leaves might be used as a good source of protein also.

As shown in Table-1.4, the small sized leaves contained 1.02% in immature, 1.50% in mature and 1.04 % in ripen stages while the large sized leaves contained 1.08 % in immature, 1.57% in mature and 1.09% of water soluble protein in ripen stages. Findings of the present study clearly indicated that like total protein the amount of water soluble protein is also increased upto mature stage and then decreased at the ripen stage.

The protein content as determined by Micro-Kjeldahl method showed considerably higher value than that given by the Lowry method. This is because Lowry method of protein was applied to an water extract of Sajna leaves and took into account, in this case, the water soluble proteins only. Further Kjeldahl method takes into account of both the protein and non- protein nitrogen.

Table-1.4: Total protein and water soluble protein contents of Sajna leaves at different maturity levels.

	Types of leaves	Stages of Maturation		
		Immature	Mature	Ripen
Total protein (%)	Small sized leaves	5.88±0.03	7.65±0.01	4.10±0.01
	Large sized leaves	5.58±0.03	7.2±0.01	4.0±0.01
Water soluble protein (%)	Small sized leaves	1.02±0.01	1.50±0.02	1.04±0.03
	Large sized leaves	1.08±0.01	1.57±0.01	1.09±0.01

1.3.8. Total Phenol content of Sajna leaves

Phenolic compounds enjoy a distribution in the plant kingdom, and they are particularly prominent in fruits and vegetables where they are important in determining color and flavour (Buren, 1970).

The amount of phenol present in two types of Sajna leaves at different maturity stages are given in Table-1.5.

In mature stages small sized leaf contained the highest amount of phenol (0.114%) followed by Large sized leaf (0.112%). It may be concluded from the results that the phenol content of Sajna leaf was increased slightly upto the mature stage and then decreased. Further, the phenol content of both the types of Sajna leaves was found to be very similar.

1.3.9. Total lipid content of Sajna leaves

Fats are concentrated form of energy and are important as carrier of certain vitamins. Lipid content of Sajna leaves are presented in Table-1.5. The present data indicated that Sajna leaves contained very little amount of lipid, so it cannot be considered as a good source of lipid.

The lipid content of Sajna leaves increased gradually at successive stages of maturation. The results indicated that small sized leaves contained slightly less amount of lipid as compared to that in large sized leaves.

Table-1.5: Total Phenol and total lipid contents of Sajna leaves at different maturity levels.

	Types of leaves	Stages of Maturation		
		Immature	Mature	Ripen
Total Phenol (%)	Small sized leaves	0.105±0.03	0.114±0.01	0.096±0.04
	Large sized leaves	0.102±0.05	0.112±0.02	0.093±0.04
Total lipid (%)	Small sized leaves	0.5±0.01	1.0±0.01	1.9±0.01
	Large sized leaves	0.7±0.02	1.12±0.02	1.95±0.01

1.3.10. Total sugar content of Sajna leaves

Total sugar content of two types of Sajna leaves are presented in Table-1.6.

Of the two types examined the large sized leaves contained higher amount of total sugar than the small sized leaves in all the maturity stages. It was also observed that the total sugar content of Sajna leaves increased sharply with the changes of maturity. The above results are similar to the findings of Abdullah *et. al.*,(1985) who reported that total sugar of banana increased with the changes of maturity. The increased in sugar content might be due to rapid conversion of starch to sugar.

1.3.11. Reducing sugar content of Sajna leaves

Table-1.6 shows reducing sugar content of Sajna leaves.

It was found that Sajna leaf contained low amount of reducing sugar and the content of reducing sugar increased gradually with the changes of maturity. The changes in reducing sugar might be due to enzymatic conversion of starch to reducing sugars and also conversion of some non- reducing sugars to reducing sugars (Ferdaus and Haque,1989).

1.3.12. Sucrose content of Sajna leaves

As shown in Table-1.6, the large sized leaves contained higher amount of sucrose at all the maturity stages.

Sucrose content in both small and large sized Sajna leaves were also found to be increased gradually with the advances in maturity. In mature stage, small sized leaf contained 1.80% while large sized leaf contained 2.89% of sucrose.

1.3.13. Starch content of Sajna leaves

Table-1.6 shows the starch content of Sajna leaves at different maturity levels.

The small sized leaves contained 3.2-6.8% while large sized leaves contained 3.9-7.8% of starch. Of the two types, large sized leaves contained higher amount of starch at all maturity stages.

The results clearly indicated that starch content of Sajna leaves decreased abruptly with the changes of maturity. The reduction of starch with the changes of maturity might be due to hydrolysis of starch by enzymes. The finding is very similar to that reported by Palmer (1971) on healthy banana.

Table-1.6: Total sugar, reducing sugar, sucrose and starch contents of Sajna leaves at different maturity levels.

	Types of leaves	Stages of Maturation		
		Immature	Mature	Ripen
Total sugar (%)	Small sized leaves	1.2±0.02	2.3±0.03	3.23±0.04
	Large sized leaves	2.3±0.01	3.3±0.05	4.5±0.05
Reducing sugar (%)	Small sized leaves	0.3±0.02	0.4±0.01	0.5±0.02
	Large sized leaves	0.216±0.02	0.317±0.02	0.417±0.02
Sucrose (%)	Small sized leaves	0.855±0.03	1.8±0.03	2.59±0.06
	Large sized leaves	1.97±0.05	2.89±0.03	3.87±0.03
Starch (%)	Small sized leaves	6.8±0.03	5.2±0.05	3.2±0.06
	Large sized leaves	7.8±0.01	6.5±0.03	3.9±0.03

1.3.14. β-carotene content of Sajna leaves

Carotenes are precursors of vitamin-A. Vitamin-A is necessary for growth and development of soft tissue through its effect upon protein synthesis and it also plays a role in the maintenance of normal epithelial structure.

The values obtained for β-carotene content of Sajna leaves are expressed in mg per hundred gm fresh leaves (Table-1.7). It was found that the content of β-carotene was higher in ripen leaves. Again, small sized leaves contained slightly higher amounts of β-carotene than the large sized leaves. Further, β-carotene was found to be increased with the changes of maturity.

1.3.15. B-Vitamins content of Sajna leaves

Vitamin-B_1

Vitamin-B_1 exists in tissue mostly in the form of thiamin Pyrophosphate (TPP) known as co-carboxylase. TPP serves as co-enzyme in the metabolism of carbohydrate, fat and protein. Thiamin is essential for growth, normal appetite, digestion and healthy nerves. Table-1.7 shows the analytical values of vitamin-B_1 content of the different types of Sajna leaves. As shown in the Table, large sized leaves content the highest amount of vitamin-B_1 (0.0055 %) than small sized leaves (0.0042 %) in mature stage. Finding of the present study clearly indicated that the amount of vitamin-B_1 is increased upto mature stage and then decreased in ripen stage.

Vitamin-B_2 (Riboflavin):

Vitamin-B_2 a crystalline pigment, is the principal growth promoting factor of the vitamin B- complex. It functions as a flavoprotein in tissue respiration. Riboflavin may be used as medicine, animal feed supplement, enriched flours, dietary supplement etc. It functions as a co-enzyme for many flavine enzymes.

Table-1.7 shows the analytical values of vitamin-B_2 content of different types of Sajna leaves at different maturity levels. As described in the Table, vitamin-B_2 content ranged between 0.00006-0.00014% in small sized leaves and 0.00008-0.00017% in large sized leaves. The present finding indicated that vitamin-B_2 of two types of leaves increased with the changes of maturity. Of the two types examined large sized leaves contained slightly higher amount of vitamin-B_2 than the small sized leaves at all the maturity stages.

1.3.16. Vitamin-C content of Sajna leaves.

Vitamin-C takes part in the formation of tissue collagen. Recent research have established the role of ascorbic acid in the conversion of folic acid to a physiologically active form tetrahydrofolic acid. Vitamin-C also involves in electron transport in the microsomal fraction. The amount of vitamin-C present in two types of Sajna leaves at different maturity stages are given in Table-1.7.

The small sized leaves contained 0.2-0.25% while the large sized leaves contained 0.22-0.28% of vitamin-C. Of the two types large sized leaves contained higher amount of vitamin-C in all maturity stages. It may be concluded from the results that the vitamin-C content of Sajna leaves was increased upto mature stage and then decreased slightly in the ripen stages.

The present data also suggested that Sajna leaves are very good sources of vitamin-C.

Table-1.7 : β-carotene, thiamine, riboflavin and ascorbic acid contents of Sajna leaves at different maturity levels.

	Types of leaves	Stages of Maturation		
		Immature	Mature	Ripen
β-carotene (%)	Small sized leaves	0.09888±0.001	0.11184±0.001	0.1289±0.002
	Large sized leaves	0.0906±0.001	0.102±0.002	0.1195±0.003
Thiamine (%)	Small sized leaves	0.003±0.0002	0.0042±0.0001	0.0021±0.0001
	Large sized leaves	0.0029±0.0002	0.0055±0.0001	0.0026±0.0001
Riboflavin (%)	Small sized leaves	0.00006±0.001	0.000102±0.0002	0.00014±0.0003
	Large sized leaves	0.00008±0.001	0.00012±0.0002	0.00017±0.0002
Ascorbic acid (%)	Small sized leaves	0.2±0.008	0.25±0.003	0.23±0.005
	Large sized leaves	0.22±0.004	0.28±0.005	0.25±0.003

1.3.17. Minerals content of Sajna leaves.

Minerals are inorganic elements exist in the body and in foods as organic and inorganic combination. In foods mineral elements are present as salt. They combined with organic compound , e. g. iron in hemoglobin. Minerals are required for the teeth and bone formation. Minute amount of mineral elements are constituents of various regulatory compounds such as, vitamins, enzymes and hormones.

For example, some enzymes require calcium for their activities as lipases and succinate dehydrogenase. Iron requiring enzymes are ferredoxin catalase, indophenol oxidase, aldehyde oxidase etc. The mineral elements present in the intra and extra cellular fluid maintained water and acid-base balance. They regulate transmission of impulses and contraction of muscles. The amount of calcium, iron and phosphorus present in the different types of Sajna leaves are given in Table-1.8.

The results indicated that the amount of calcium decreased gradually with the advance in maturity. Sajna leaves of small sized contained higher amount of calcium (0.0084%) in immature stage while that of large sized contained lowest amount (0.0052%) in ripen stage. Of the two types examined, large sized leaves contained slightly higher amount of iron (0.0070%) than the small sized leaves (0.0067%) in immature stage. The present finding also indicated that iron content of leaves decreased gradually with the advance in maturity.

From the table it was clear that small sized leaves contained higher amount of phosphorus than the large sized leaves at each stages of maturation. Findings of the present study clearly indicated that the amount of phosphorus increased very rapidly with the changes of maturity. It might be due to the increased rate of production of energy rich phosphate compound. In conclusion, the present data clearly suggested that Sajna leaves may be used as a good sources of minerals such as calcium, iron and phosphorus.

Table-1.8: Calcium, iron and phosphorus contents of Sajna leaves at different maturity levels.

	Types of leaves	Stages of Maturation		
		Immature	Mature	Ripen
Calcium (%)	Small sized leaves	0.0084±0.0001	0.0075±0.0002	0.0063±0.0003
	Large sized leaves	0.0071±0.0001	0.0063±0.002	0.0052±0.0005
Iron (%)	Small sized leaves	0.0067±0.0001	0.0062±0.0001	0.0055±0.0001
	Large sized leaves	0.007±0.0001	0.0065±0.0002	0.0059±0.0002
Phosphorus(%)	Small sized leaves	0.046±0.002	0.053±0.003	0.065±0.006
	Large sized leaves	0.035±0.002	0.041±0.002	0.057±0.001

CHAPTER-2

Activities of some hydrolytic and oxidative enzymes in two different types of Sajna leaves at different maturity levels

2.1. INTRODUCTION

Sajna leaves as well as fruits are the cheap vegetables of Bangladesh. It was found from the present data that the physico-chemical properties of Sajna leaves are greatly affected with the changes of maturity (Shahanaz Khatun *et. al.*, 2003). Proteolytic and hydrolytic enzymes may play some physiological roles during maturation and senescence of fruit (Hasinaga *et. al.*, 1983; Desai and Deshpande; 1978b). Dilley (1970) suggested that the dramatic physical and chemical changes attending ripening occur as a result of catabolic and anabolic processes might be enzyme directed processes.

In this study the activities of amylase, protease, cellulase, invertase, ascorbic acid oxidase, polyphenol oxidase and peroxidase are measured at different maturity stages of Sajna leaves.

2.2. MATERIALS AND METHODS

2.2.1. Preparation of crude enzyme extract

At first 10 g of Sajna leaves were cut into small pieces and grinded in a morter with pestle and then homogenized well with cold 0.1M phosphate buffer of respective pH (for amylase: pH 6.7, for invertase and protease: pH 7.0, for polyphenol oxidase, peroxidase and ascorbic acid oxidase: pH 6.0) while for the measurement of cellulase activity 0.1M sodium acetate buffer, pH 5.2 were used. Then filtered the extract through a double layer of muslin cloth. After centrifugation at 4,000 g for 5 minutes the supernatant was used as crude enzyme extract.

2.2.2. Measurement of amylase activity

Amylase activity was assayed following the method as described in laboratory Manual in Biochemistry (Jayaraman, 1981). One percent starch solution was used as substrate. The amylase activity was measured by estimating the release of maltase. The amount of maltose released was calculated from the standard curve prepared with maltose. One unit of amylase activity was defined as the amount required for liberating 1μg of maltose per min at 37°C.

2.2.3. Measurement of protease activity

The protease activity was measured following the method of Kunitz (1947). The milk protein, casein was used as a substrate. The activity is determined by detecting the release of amino acid (tyrosine). The amount of tyrosine released was calculated from the standard curve constructed with tyrosine. One unit of protease activity was defined as the amount required for liberating 1μg of tyrosine per min at 45°C.

2.2.4. Measurement of invertase activity

Invertase activity was assayed following the modified method as described in methods in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). Sucrose was used as substrate. The invertase activity was measured by estimating

the release of glucose. The amount of glucose released was calculated from the standard curve prepared with glucose. One unit of invertase activity was defined as the amount required for liberating 1μg of glucose per min at 30°C.

2.2.5. Measurement of cellulase activity

The cellulase activity was measured following the procedure as described in method in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). Carboxymethyl cellulose (CMC) was used as substrate. Cellulase activity was measured by estimating the release of reducing sugar by cellulase. The amount of reducing sugar released was determined by dinitrosalicylic acid method (Miller,1972). One unit of cellulase activity was defined as the amount of enzyme required for liberating 1μg of reducing sugar per min at 37°C.

2.2.6. Measurement of ascorbic acid oxidase activity

Ascorbic acid oxidase activity was measured following the procedure as described in methods in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). In this process ascorbic acid was used as substrate. The enzyme activity was measured by determining the residual ascorbic acid in the reaction mixture. One unit of enzyme activity was defined as a change in absorbance of 0.01 at 265 nm per min (Kaul and Munjal, 1980).

2.2.7. Measurement of polyphenol oxidase activity

Polyphenol oxidase activity was measured following the procedure as described in methods in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). In this process catechol was used as substrate. One unit of enzyme activity was defined as a change in absorbance of 0.001 at 495nm per min.

2.2.8. Measurement of peroxidase activity

Peroxidase activity was measured following the procedure as described in methods in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). In this method pyrogallol was used as substrate. In presence of H_2O_2 pyrogallol is oxidized to coloure derivative. The amount of purpurogalin formed during the reaction can be followed in a spectrophotometer.One unit of enzyme activity was defined as the amount of purpurogalin formed per min under the assay condition.

2.3. RESULT AND DUSCUSSION

2.3.1. Activity of amylase in Sajna leaves.

Amylase is a hydrolytic enzyme which hydrolyses starch to yield monomeric carbohydrate. The amount of amylase present in the Sajna leaves is presented in the Table-2.1.

Of the two types analyzed, large sized leaves contained the higher amount of amylase activity (24.3 unit g^{-1}leaf) than the small sized leaves (19.55 unit g^{-1} leaf) in the mature stage. The present finding also indicated that the activity of amylase increased upto mature stage and then decreased sharply in ripen stage. Similar trends was also reported by Desai and Deshpande (1978b); Nabeesa and Unnikrishnan(1988);Mao and Kinsella (1981) and Garcia *et al* (1988) in case of banana. The present data clearly revealed the involvment of amylase in starch degradation during ripening stages of leaves.

2.3.2. Activity of protease in Sajna leaves.

Protease is a protein hydrolyzing enzyme, which act on proteinaceous substances releasing amino acids and amides. Table-2.1 shows the protease activities of two types of Sajna leaves at different stages of maturity. The present data clearly indicated that the protease activity of both types of Sajna leaves increased remarkbly with the changes in maturity and the activity is highest at the ripen stage. The protease activities of leaves was found to be varied between 1.2-4.1 unit $g^{-1}$of leaf at different maturity stages.

Hashinaga *et. al.* (1986) reported that protease activity (KFP-1) increased in KiwiFruit flesh during ripening. Increased protease activity was also observed in passion fruit juice during maturation (Hashinaga *et. al.* 1978). Increased protease activity during ripening stage may be attributed to protein catabolism which is related to leaf senescence (Diley, 1970).

Table-2.1: Activities of amylase and protease in Sajna leaves at different maturity stages.

	Types of leaves	Stages of Maturation		
		Immature	Mature	Ripen
Amylase (unit g^{-1})	Small sized leaves	10.0±0.001	19.550±0.001	12.18±0.002
	Large sized leaves	11.33±0.003	24.3±0.003	14.6±0.002
Protease (unit g^{-1})	Small sized leaves	1.2±0.002	3.01±0.001	4.10±0.001
	Large sized leaves	1.89±0.001	3.10±0.001	4.05±0.002

2.3.3. Activity of cellulase in Sajna leaves.

Cellulase is a hydrolytic enzyme, produced by the bacteria in the digestive tracts of animal, and is responsible for release of glucose from cellulose. The cellulase activities of two types of Sajna leaves are given in the Table-2.2.

It was found that small sized Sajna leaves contained 18.4 units whereas large sized leaves contained 17.2 units of cellulase per g leaf at mature stage.

The present finding also indicated that the cellulase activity of Sajna leaves increased upto mature stage thereafter the activity was reduced drastically.

2.3.4. Activity of invertase in Sajna leaves.

Invertase is a hydrolytic enzyme which hydrolyzes sucrose to glucose and fructose. Invertase activity of Sajna leaves is shown in Table-2.2.

From the Table it was clear that the small sized leaf contained slightly higher amount of invertase activities in all the maturity stages. The present finding indicated that the invertase activity in two types of Sajna leaves increased slightly upto mature stage and thereafter decreased. Further Sajna leaves contained very low amount of invertase activity.

Table-2.2: Activities of cellulase and invertase in Sajna leaves at different maturity stages.

	Types of leaves	Stages of Maturation		
		Immature	Mature	Ripen
Cellulase (unit g^{-1})	Small sized leaves	7.0±0.002	18.4±0.001	3.1±0.003
	Large sized leaves	6.5±0.002	17.2±0.001	2.95±0.002
Invertase (unit g^{-1})	Small sized leaves	0.09±0.002	0.13±0.003	0.043±0.003
	Large sized leaves	0.06±0.001	0.10±0.001	0.03±0.001

2.3.5. Activity of ascorbic acid oxidase in Sajna leaves.

This enzyme is widely distributed throughout the plant kingdom. Ascorbic acid oxidase catalyzes the direct oxidation of ascorbic acid by molecular-oxygen, according to the equation.

$$\text{Ascorbic acid} + 1/2\ O_2 \longrightarrow \text{Dehydroascorbic acid} + H_2O$$

The ascorbic acid oxidase content of Sajna leaves is shown in Table-2.3. It was found that ascorbic acid oxidase activity of Sajna leaves increased rapidly upto mature stage and thereafter decreased sharply in both types of Sajna leaves. Both types of Sajna leaves contained significant amount of ascorbic acid oxidase at mature stage (34-35 units per g of leaf).

2.3.6. Activity of polyphenol oxidase in Sajna leaves.

Polyphenol oxidase is also known as phenoloxidase, tyrosinase, dopaoxidase, catecholoxidase and potatooxidase. This enzyme catalyzes the oxidation of monophenols and orthodiphenols. Monophenols particularly tyrosine, and ρ-

cresol, orthodiphenols such as adrenaline, pyrogallol and substituted catechols are important substrates of the enzyme. The equation, which catalyzed by polyphenol oxidase is:

$$\text{Catechol} + O_2 \longrightarrow 2\ \text{O-Benzoquinone} + 2H_2O$$

Table-2.3 shows polyphenol oxidase activities of two types of Sajna leaves at different stages of maturity. The polyphenol oxidase activity of Sajna leaves increased rapidly with the advancement of maturity in both types upto mature stage and thereafter decreased in ripen stage. Large sized Sajna leaf contained 80 units whereas small sized leaf contained 50 units of polyphenol oxidase g^{-1} leaf at mature stage.

2.3.7. Activity of peroxidase in Sajna leaves.

Peroxidase catalyzes the oxidation of various "hydrogen donors" like ρ-cresol, benzidine, ascorbic acid, nitrate and cytochrome in the presence of H_2O_2.

The reaction may be presented as :

$$H_2O_2 + 2AH \longrightarrow 2A + H_2O$$

Peroxidase are widely distributed in the plant kingdom. Table-2.3 shows peroxidase activity of two different sized Sajna leaves at different maturity stages.

As found, large sized leaf contained highest amount of peroxidase (37.6 units g^{-1} leaf) in ripen stage while small sized leaf contained lowest amount (15.2 units g^{-1} leaf) in immature stage.

The present results clearly demonstrated that the activity of peroxidase increased rapidly with the changes of maturity.

Table-2.3: Activities of ascorbic acid oxidase, polyphenol oxidase and peroxidase in Sajna leaves at different maturity stages.

	Types of leaves	Stages of Maturation		
		Immature	Mature	Ripen
Ascorbic acid oxidase (unit g^{-1})	Small sized leaves	28.5±0.01	34.0±0.011	17.0±0.01
	Large sized leaves	30.1±0.02	35.0±0.02	20.0±0.03
Polyphenol oxidase (unit g^{-1})	Small sized leaves	25±0.02	50±0.03	20±0.03
	Large sized leaves	40±0.02	80±0.02	30±0.03
Peroxidase (unit g^{-1})	Small sized leaves	15.2±0.01	31.0±0.02	36.5±0.02
	Large sized leaves	16.0±0.02	33±0.01	37.6±0.01

CHAPTER-3

Purification and characterization of polyphenol oxidase from large sized Sajna leaves at mature stage.

3.1. Introduction

Polyphenoloxidase (PPO) is a copper-containing enzyme which catalyzes the hydroxylation of monophenols to o-diphenols and the oxidations of o-dihydroxyphenol to o-quinols utilizing molecular oxygen. These quinones are highly reactive, electrophilic molecules which covalently modify and cross link to a variety of cellular constituents. The reaction is very important in maturation and ripening process of fruits and vegetables since it removes astringency by converting soluble phenolic compounds into insoluble ones through oxidation and polymerization process. It is present in almost all the plant species either as active or latent. Polyphenoloxidase has been purified from mushrooms (Smith and Krueger, 1962; Bouchillous *et al.*, 1963) and potato tuber (Jonna and Paul, 1996) and Bartlett pears (Tate *et al.*, 1964). It was found in our laboratory that large sized Sajna leaves at mature stage contained the highest amount of PPO activity (Khatun *et al*, 2003). The present chapter describes the Purification, Charscterization and effect of physico-chemical agents on the stability of PPO from large sized Sajna leaves at mature stage.

3.2. MATERIALS AND METHODS

Freshly harvested large sized Sajna leaves at mature stage were collected from the selected Sajna tree. Catechol, DEAE-cellulose and CM-cellulose were obtained from Pharmacia Fine Chemicals Co. Sweden. Trypsin inhibitor, BSA, α-amylase and SDS were purchased from Sigma Chemicals Co. USA. All other reagents used were of analytical grade.

3.2.1. Measurement of enzyme activity

Procedure was followed as described in methods of Physiological Plant Pathology (Mahadevan and Sridhar, 1982) using catechol as substrate (Chapter-2, 2.2.7.).

3.3. Purification of enzyme

3.3.1. Preparation of crude enzyme extract

All the operations were performed at 4^0C. Sajna leaves (155 gm) were cut into small pieces and grinded into paste with cold 0.1M phosphate buffer, pH-6.0 and finally homogenized well into a uniform slurry using a tissue homogenizer. The slurry was filtered through double layer of cheese cloth and the filtrate was further clarified by centrifugation at 6000 g for 15 min and the clear supernatant was concentrated to about 1/8th of its original volume by the sucrose. The concentrate was then dialyzed against 10mM phosphate buffer, pH-7.5 overnight. The dialyzate was then centrifuged at 7000 g for 8 min and the clear supernatant was used as crude enzyme extract.

3.3.2. DEAE - Cellulose Column Chromatography

The crude enzyme extract was loaded into a DEAE-cellulose column, which was previously equilibrated with 10mM phosphate buffer, pH-7.5 and the protein was eluted from the column by the same buffer with stepwise increasing concentrations of NaCl. Enzyme activity and protein concentration were measured at intervals of one fraction (3ml/tube).

3.3.3. CM - Cellulose Column Chromatography

The enzyme active fraction, obtained from DEAE- cellulose column, was collected and dialyzed against 10mM sodium phosphate buffer; pH 6.5 for 24 hours. After centrifugation the clear supernatant was loaded into a CM- cellulose column. The separation was achieved by stepwise elution of protein from the column with increasing concentrations of NaCl in the same buffer. Enzyme activity and protein concentration were measured at intervals of one fraction (3ml/tube).

3.3.4. Estimation of protein concentration

Protein concentration was routinely analyzed by the absorbance at 280 nm by the method of Lowry *et al.* (1951).

3.3.5. Polyacrylamide disc gel electrophoresis

Purity of the enzyme was checked by polyacrylamide disc gel electrophoresis following the method of Ornstein (1964).

3.3.6. Molecular weight determination

Molecular weight of PPO under non-denaturing condition was determined by gel filtration on Sephadex G-150 column (0.9 x 90 cm) as described by Andrews (1965). Trypsin, egg albumin, bovine serum albumin, β-galactosidase, and β-amylase were used as marker proteins. Furthermore, the molecular weight under denaturing condition as well as the sub-unit structure was determined by SDS-PAGE (Weber and Osborn, 1969).

3.3.7. Ultraviolet absorption spectrum

It was recorded in aqueous solution with Shimadzu UV-180 double beam spectrophotometer at room temperature.

3.3.8. Determination of pH optimum

The activity of PPO was determined at different pH-values ranging from pH-3.0 to 9.5 (20mM Na-acetate buffer, pH: 3.0-4.5; 10mM phosphate buffer, pH 5.0-7.0

and 10mM tris HCl buffer pH 7.5-9.5,) at 32°C following the procedure as described earlier (Chapter 2).

3.3.9. Determination of temperature optimum

The activity of PPO was determined at different temperature ranging from 5 to 70°C, using 50mM phosphate buffer; pH 6.2. The enzyme solution was incubated for 20 min at respective temperature and after cooling the activity was measured following the procedure as described earlier (Chapter 2).

3.3.10. Determination of activity of PPO in presence of different chemicals and metallic salts

To the PPO solution (O.D: 0.25- 0.30 at 280 nm) (0.5 ml), were added chemicals and metallic salts of different concentrations and incubated for 10 min at 20°C and enzymic activity was measured following the procedure as described earlier (Chapter 2).

3.4. RESULTS

3.4.1. Purification of enzyme

As shown in Fig. 3.1 the proteins of the crude enzyme extract were eluted from the DEAE column in two major peaks, F-1 and F-4 and four other minor peaks (F-2, F-3, F-5 and F-6). The major peak, F-1 was eluted from the column by the buffer only while the five other fractions were eluted from the column stepwise with increasing concentration of NaCl. Of these fractions, it was found that only the major fraction F-4 contained the PPO activity. The fraction F-4 as indicated by solid bar was collected and its purity was checked. It was found to contain more than one band on the gel (Fig. 3.3).

So for further purification, this fraction was dialyzed against 10mM sodium phosphate buffer, pH 6.5 for 24 hours and applied to a CM-cellulose column. As shown in Fig.3.2, the components of F-4 fraction were separated into one major peak (F-4a) and two other minor peaks, (F-4b and F-4c). Of these fractions, the major fraction F-4a, which was eluted by the buffer only, while the other fractions F-4b and F-4c were eluted by the buffer containing 0.05 M and 0.1M NaCl respectively. Of these three fractions only F-4a contained the PPO activity. The fraction, F-4a, as indicated by solid bar was pooled and its purity was checked.

3.4.2. Polyacrylamide gel electrophoresis

From Fig.3.3 it might be concluded that the fraction F-4a contained pure Polyphenoloxidase since it gave single band on the gel.

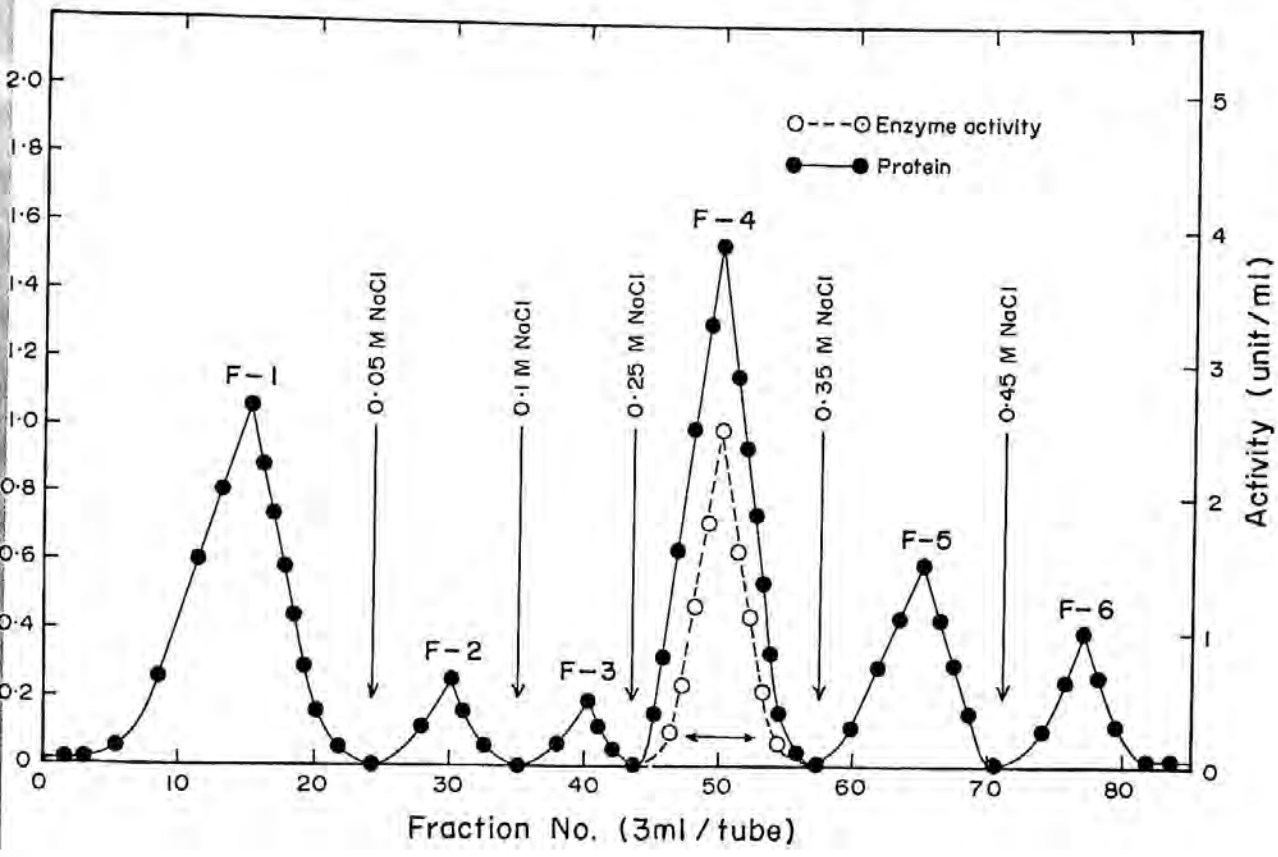

Fig. 3.1: Stepwise elution profile of PPO from DEAE-cellulose column. Crude enzyme extract (155 mg protein) was applied to a column (1.5×28cm) pre-equilibrated with 10mM phosphate buffer, pH 7.5 and the protein were eluted from the column stepwisely with the same buffer containing different concentration of NaCl with a flow rate of 25 ml/ hour.

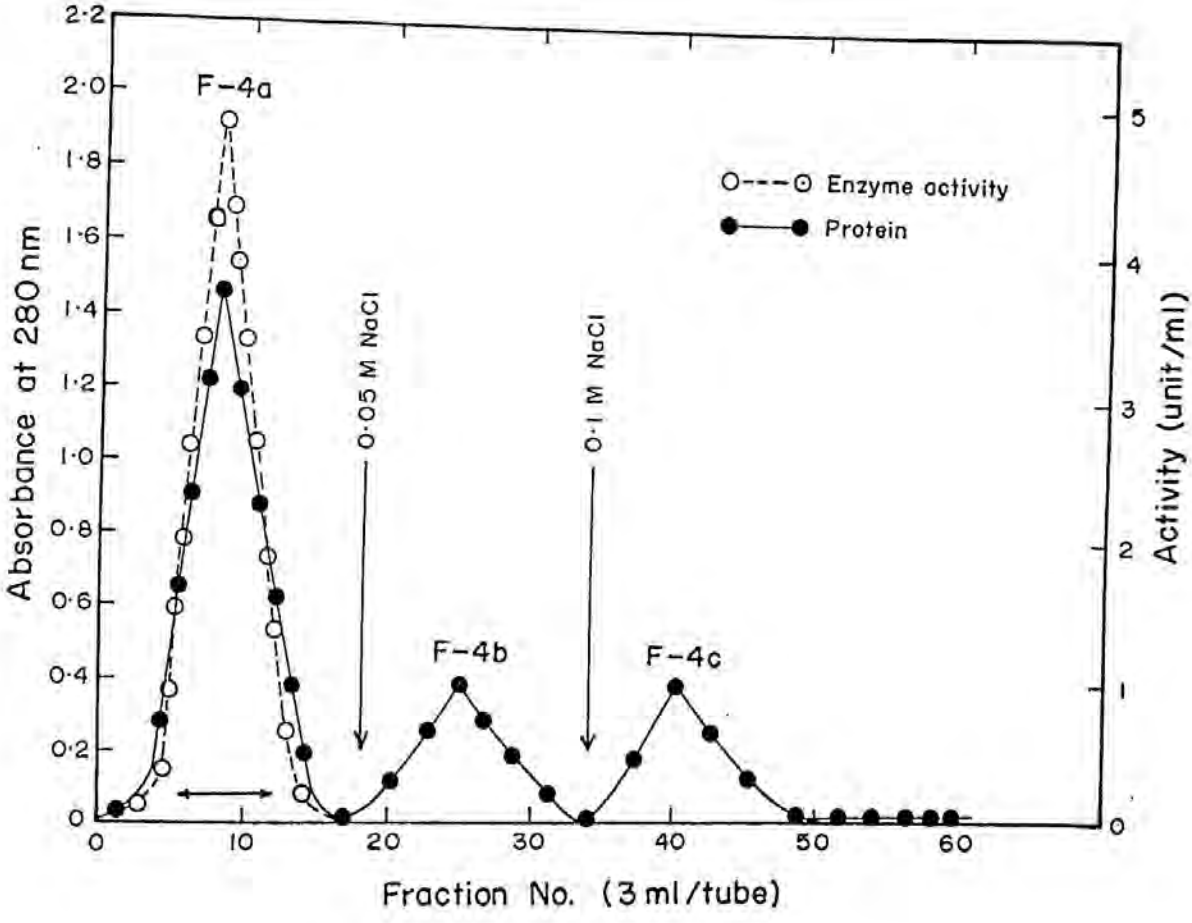

Fig. 3.2: Stepwise elution profile of F-4 fraction on CM-cellulose column. Protein (4.25 mg) was applied to a column (0.5×15cm) pre-equilibrated with 10mM phosphate buffer, pH 6.5 and eluted with the same buffer containing different concentration of NaCl with a flow rate of 20 ml/ hour.

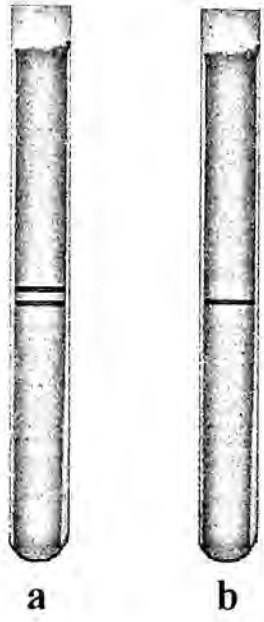

Fig.3.3: Polyacrylamide disc gel electrophoretic pattern of the protein at room temp. pH 8.3 on 7.5 % Polyacrylamide gel.

Staining reagent : 1 % amido black.

Protein concentration : 50μg.

a : F-4 fraction (after DEAE- cellulose chromatography)

b : F-4a fraction (after CM- cellulose chromatography).

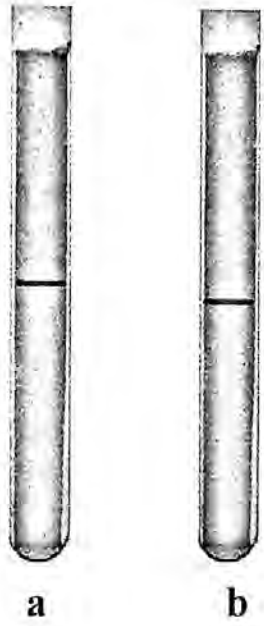

Fig. 3.4: SDS-Polyacrylamide disc gel electrophoresis of PPO under reducing and non- reducing conditions on 10% gel.

Staining reagent: 0.1 % Coomassic brilliant blue R-250

Protein concentration: 50μg.

a) Absence of β-mercaptoethanol

b) Presence of β-mercaptoethanol

The extent of purification, recovery and yield of enzyme at each step are summarized in Table 3.1.

It was found that the specific activity of the enzyme increased in each of the subsequent purification step. Although the yield was only about 25% and over 95% of the extracted protein was removed during purification steps but the enzyme was purified with an increase in purification fold of about 28.

Table 3.1: Summary of purification of PPO enzyme

Steps	Total protein (mg)	Total activity (units/ml)	Specific activity (unit/mg)	Activity yield (%)	Purification fold
Crude extract	155	109	0.703	100	1
DEAE-cellulose ion exchange chromatography	4.25	56.5	13.29	51.83	18.90
CM-cellulose ion exchange chromatography	1.4	27.2	19.4	24.95	27.59

3.4.3. Characterization of enzyme

The MW of PPO was calculated to be about 56,000 by Gel filtration. On the other hand, the MW of PPO by SDS-PAGE was found to be 55,500 (Fig. 3.4). This small discrepancy in MW might be withiin the error range of SDS-PAGE. Furthermore, the MW obtained in presence of β-mercaptoethanol was almost the same as that found in absence of β-mercaptoethanol indicating that the enzyme is consisted of a single polypeptide chain (Fig. 3.4).

The purified PPO in aqueous solution gave absorption maximum at 279 nm and minimum around 238 nm (F.g.3.5). As shown in Fig.3.6 the activity of PPO was greatly affected with changes in pH and pH-activity profile showed a characteristic bell-shaped curve. It was found from the curve that the enzyme gave

about 80% activity at pH 5.0 and at pH 7.5 with an optimum pH value of 6.2. Beyond these pH ranges, both at the acidic as well as alkaline sides the activity of the enzyme decreased sharply and the enzyme lost more than 80% of its activity at pH 3.0 and at pH 9.5.

The activity of PPO was also found to be remarkably affected by the changes in temperature. As shown in Fig. 3.7, the activity of PPO increased gradually with rise in temperature and the enzyme gave maximum activity at 32°C. With further rise in temperature the activity decreased rapidly and more than 95% of its activity was lost at 68°C.

The Km value of the PPO against catechol as substrate, calculated from Lineweaver-Burk double reciprocal plot was 0.047 M.

3.4.4. Effect of Chemicals and metallic salts on the activity of PPO

As shown in Table-3.2 the activity of PPO decreased gradually with higher concentration of Chemicals and the enzyme lost its activity completely at 0.004M, 0.4M, 0.01M and 0.01M EDTA, ascorbic acid, KCN and $NaHSO_3$ respectively. Further metallic salts such as Cu^{2+} and Ca^{2+} increased the activity significantly, while Mg^{2+} salt decreased the activity remarkably. On the other hand Hg^{2+} and Fe^{2+} salt have moderate inhibitory effect on the activity of PPO.

Table 3.2: Effect of ascorbic acid, KCN, EDTA & Sodium bisulfite on the activities of PPO

Concentration of Ascorbic acid (M)	0	0.002	0.005	0.01	0.05	0.1	0.2	0.3	0.4
Relative activities (%) of PPO	100	92.2	80.3	49.22	28.10	10.0	5.0	1.3	0
Concentration of KCN (M)	0	0.001	0.002	0.005	0.01				
Relative activities (%) of PPO	100	85.5	40.0	16.12	0				
Concentration of EDTA (M)	0	0.0005	0.001	0.002	0.003	0.004			
Relative activities (%) of PPO	100	91	70.1	42.2	17.4	0			
Concentration of $NaHSO_3$ (M)	0	0.001	0.002	0.004	0.006	0.007	0.008	0.01	
Relative activities (%) of PPO	100	92.5	82.2	69.1	55.5	35.9	8.5	0	

Table 3.3: Effect of metallic salts on PPO activity

Test salts	Concentration (mM)	PPO activity(%)
None	-	100
$CaCl_2$	0.001	118
	0.002	107
$CuCl_2$	0.001	125
	0.002	117
$MgCl_2$	0.001	55
	0.002	35
$HgCl_2$	0.001	85
	0.002	80
$FeCl_2$	0.001	80
	0.002	74

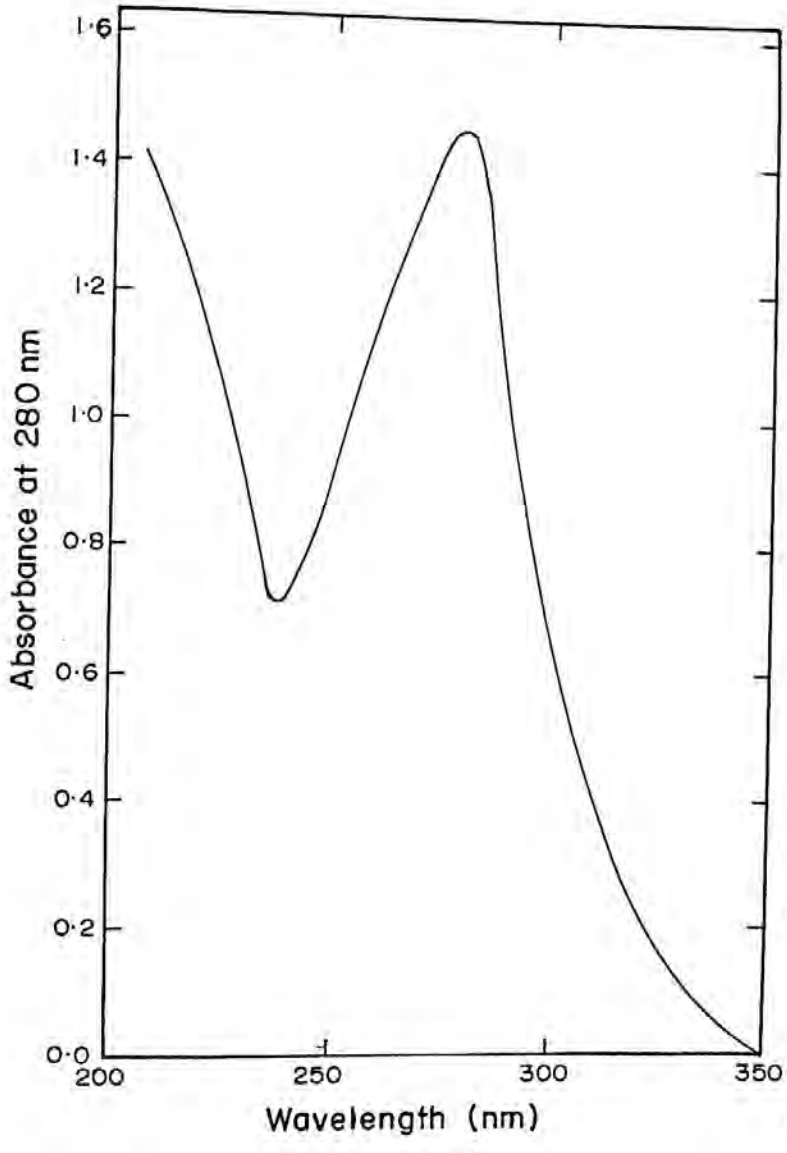

Fig.3.5: Ultraviolet absorption spectrum of the purified PPO enzyme.

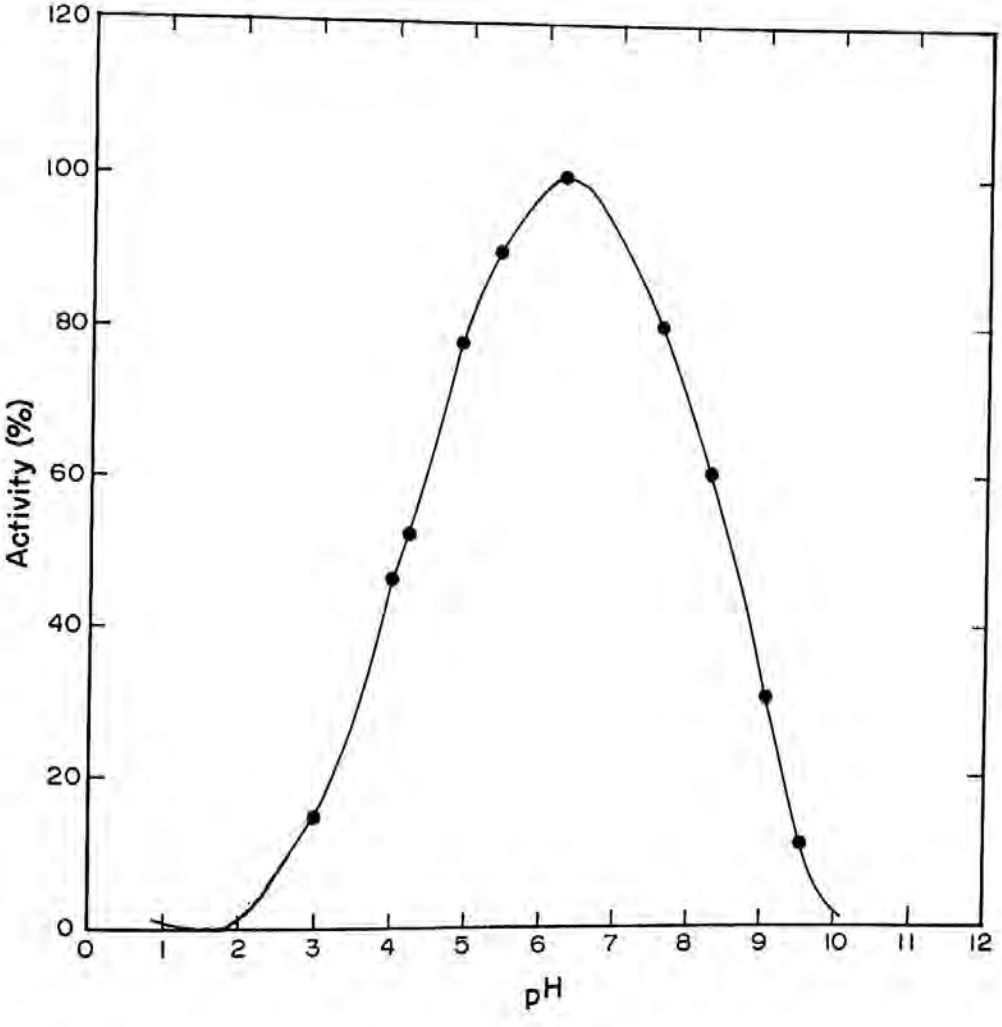

Fig.3.6: Effect of pH on Sajna leaves PPO activity.

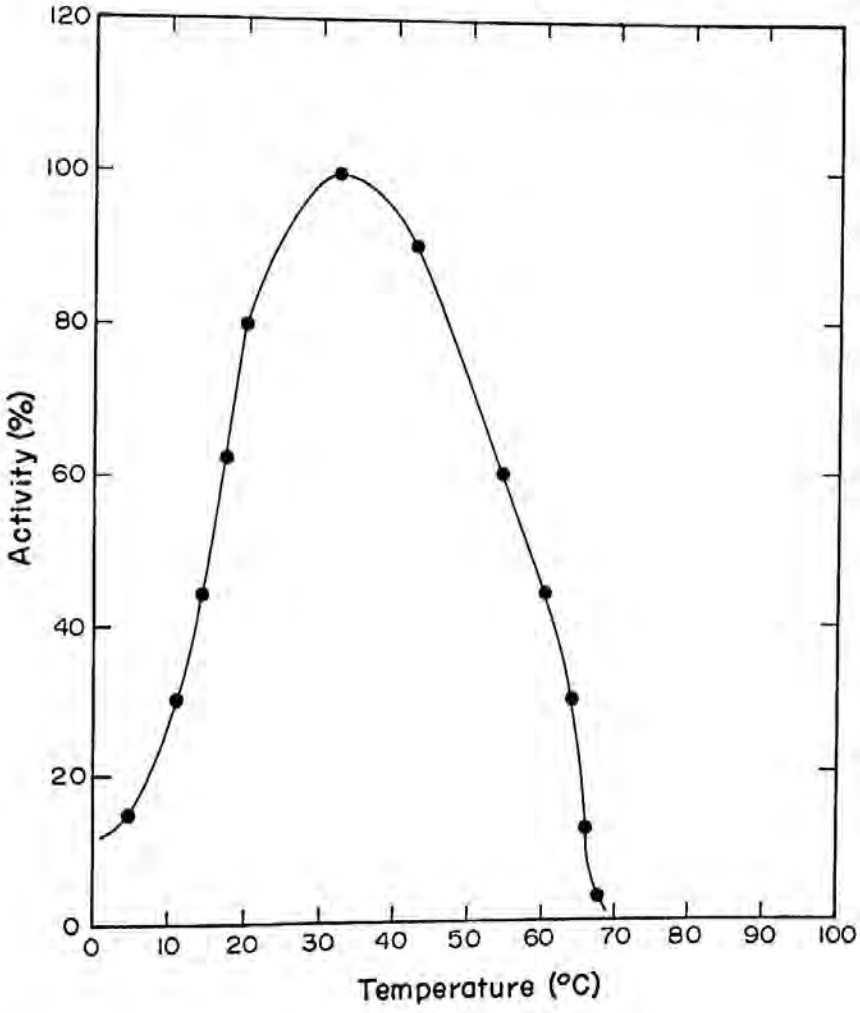

Fig. 3.7: Effect of temperature on Sajna leaves PPO activity.

3.5. Discussion

Sajna leaves are the vegetable, available almost throughout the year in our country. Pressed juice of the leaves of this plant show strong antibacterial activity and in the present study PPO was found to be very much active in the leaf juice of mature stage. Many reports were published on the purification and characterization of PPO from different sources. Some reports have shown PPO as isomeric form having MW within the range of 35-116 kDa (Owusu-Ansah, 1989; Ganesa *et al.*, 1992) but we purified homogeneous form of PPO from Sajna leaves with MW of about 56,000, which was close to the MW of PPO isolated from potato tuber (Joanna and Paul, 1996). The yield of purified PPO from Sajna leaves was found to be only about 25%, which might be due to denaturation of protein during lengthy purification periods. Further, in our laboratory it was found also that Sajna leaves contained significant amount of phenolic compounds. It was reported that the yield of PPO from guava was improved about 20 fold by adding polyvinylpyrolidine (PVP) to the extracting buffer (Mowlah and Itoo, 1982), but we could not use PVP in this study.

The activity of PPO was found to be decreased significantly at the acidic as well as alkaline pH- regions indicating the ionization of groups located in or near active sites at these pH regions. The optimum pH for purified PPO showed uniqueness with that isolated from bartlett pears (Tate *et al.*, 1964).

The PPO, was found to be very labile to temperature as compared to that from other sources and gave maximum activity at 32°C. The rapid decrease in activity above high temperature might be due to changes in secondary and tertiary structure or destruction of active site of enzyme at higher temperature. The kinetic parameter, Km of Sajna leaf PPO, obtained in present study showed a unique similarity with that of PPO from bartlett pears (Tate *et al.*, 1964).

The purified PPO lost its activity completely in the presence of 4mM EDTA, suggesting the necessity of metal ions for the activity of enzyme which was also

confirmed from the findings that the activity of PPO increased in the presence of metallic salts of Cu^{2+} and Ca^{2+}. The activity of PPO from carrot was also reported to be activated by Ca^{2+} ion (Soderhall,1995). The activity of Sajna leaves PPO was abolished completely in presence of ascorbic acid, KCN and $NaHSO_3$, suggesting that these chemicals may directly act on the active site of enzyme. Golan-Goldhirsh *et al.*, (1992) suggested that ascorbic acid and copper ions catalyze a free radical reaction that oxidizes the imidazole group of the histidine residues that ligand the active site coppers, thereby inactivating the enzyme.

CHAPTER-4

Purification and characterization of Proteins from small sized Sajna leaves at mature stage.

4.1. Introduction

Lectin, isolated chiefly from plants, bacteria, fungi, invertebrates and vertebrates are non-immunoglobulin type carbohydrate recognition molecules which can be involved in hemagglutination, lymphocyte transformation, inactivation of certain types of tumour cells and precipitation of certain polysaccharides and glycoproteins (Lis and Sharon, 1986; Goldstin and Hayes, 1978). Plant lectins isolated from the wide varieties of plants have recently been attracted great interest because of their remarkable effects in biological actions. More recently, the lectins as the carbohydrate binding proteins have been investigated and utilized in various biochemical fields. Some authors have described the purification and chemical properties of lectins from many kinds of plant materials, such as *Phaseolus vulgaris* seeds (Itoh *et. al.*, 1980), *Viscum album* L (Franz *et. al.*, 1981), *Lathyrus sativus* seeds (Kolberg and Sletten, 1982),*Vicia unijuga* leaves (Yanagi *et. al.*, 1990) etc. Lectins are being used increasingly to probe the structure of carbohydrates on the surfaces of normal and malignant cell (Liener *et. al.*, 1986).

Most plant tissues contain one lectin, but in some cases two or more lectins that differ in their sugar specificities and other properties are present. They are classified into a small number of specificity groups (mannose, galactose, N-acetylglucoseamine, N-acetylgalactoseamine and N-acetylneuraminic acid) according to the monosaccharide that is the most effective inhibitor of the agglutination of erythrocytes or precipitation of carbohydrate containing polymers by the lectin.

The present study was, therefore, undertaken with the objective of purifying the proteins from the small types Sajna Leaves at mature stage in the biologically active form.

Furthermore, these proteins were also characterized, with respect to the molecular weight, neutral sugar composition, cytoagglutination, toxicity etc.

4.2. Materials and Methods

4.3. Chemicals

Sephadex G-75, Sephadex G-150 and Sepharose-4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose was purchased from Sigma Chemical Co., USA. Molecular weight markers were the products of Fluka Biochemica, Switzerland. All the other reagents used were of analytical grade. Unless otherwise specified, all the operations were performed at 4^0C.

4.4.Collections of leaves

The mature leaves of *Moringa oleifera* Lam. Commonly known as Sajna in our country were collected from Kazla of Rajshahi district, Rajshahi, Bangladesh.

4.5. Choice of Extracting Solvent

Proteins from fresh Sajna leaves were extracted with five different extracting solvents under identical conditions. The suitable extracting solvent was selected from the ratio of absorbance at 280 nm and 260 nm as reported by Clark and Switzer (1977).

Table-4.1: Extraction of crude proteins from Sajna leaves in different extracting solvents.

No. of Observations	Extracting media	Amount of leaf	Ratio of O.D. at 280 nm & 260 nm
1	1% CH_3COOH	1gm	0.9402
2	Tris-HCl buffer,pH - 8.4	1gm	0.9700
3	Phosphate buffer, pH-7.4	1gm	0.9611
4	Distilled water	1gm	0.9701
5	0.2M NaCl in distilled water, pH-6.5	1gm	0.9809

Distilled water containing 0.2M NaCl, pH-6.5 was used as extracting solvent for preparation of crude protein extract from Sajna leaves as the highest ratio of absorbance at 280 nm and 260 nm was found (Table-4.1).

4.6. Preparation of Crude Protein Extract

Sajna leaves (155 g) were grinded in a morter and pastle, at that time very small amount of polyvinylpyrolidine was added (to avoid the phenolic compounds and other coloring substances) and was mixed uniformly with pre-cooled distilled water containing 0.2M NaCl, pH-6.5 (6ml/gm of leaf) and kept overnight at 4^0C with occasional gentle shaking. The suspension was then centrifuged at 8,000 g, 4^0C for 15 minutes. The clear supernatant was collected and adjusted to 100% saturation by adding solid ammonium sulfate. The resulting precipitate was collected by centrifugation, dissolved in minimum volume of pre-cooled distilled water and dialyzed against distilled water for 24 hours with three changes and against 10 mM Tris-HCl buffer, pH-8.4 for 12 hours at 4^0C. After centrifugation the clear supernatant was used as crude protein extract.

4.7. Purification of Proteins

4.7.1. Gel Filtration

Gel filtration was carried out on Sephadex G-75 column. The crude protein extract was loaded onto the Sephadex G-75 column previously equilibrated with 10 mM Tris-HCl buffer, pH-8.4 and the proteins were eluted with the same buffer at 4^0C.The different fractions were collected and checked for hemagglutinating activity. Absorbance at 280nm of each fraction as well as protein concentration by Folin- Lowry method (Lowry *et. al.*, 1951) were also measured.

4.7.2. DEAE-Cellulose Chromatography

The active protein fraction obtained after gel filtration was dialyzed against distilled water for 12 hours and against 10 mM Tris-HCl buffer, pH-8.4 for overnight. It was then applied to DEAE-cellulose column previously equilibrated

with 10 mM Tris-HCl buffer, pH-8.4. The absorbed proteins were eluted from the column by the linear gradient elution of NaCl (0.0- 0.3 M) in the same buffer as well as stepwise with the buffer containing different concentrations of NaCl. Analysis of each fraction was carried out similarly as described above.

4.7.3. Affinity Chromatography

For further purification of the protein, the active protein fractions obtained after DEAE-Cellulose Chromatography were dialyzed separately against distilled water for 12 hours and against 5mM phosphate buffer saline, pH-7.2 for overnight and then were applied individually to Sepharose 4B column previously equilibrated with the same buffer at 4^0C.The column was first eluted by the buffer and then with the same buffer containing 0.2 M galactose. Analysis of each fraction was also performed similarly as described above.

4.8. Purity test:

The homogeneity of the different fractions obtained after affinity chromatography was judged by 10% SDS-PAGE according to the method of Laemmli (1970) as modified by Smith (1995).

4.9. Characterization of Proteins

4.9.1. Molecular Weight Determination

a) Gel Filtration

The molecular weight of the purified proteins were determined by the gel filtration on Sephadex G-150 coloum using β-amylase, β-D- galactosidase, bovine serum albumin, egg albumin and lysozyme as marker proteins according to the method of Andrews (1965).

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

The molecular weight of the purified proteins were determined by the method of Sodium dodecyl sulfate polyacrylamide gel electrophoresis. The method was described by Laemmli (1970) as modified by Smith (1995). The molecular weight markers employed for SDS-PAGE were same as those used for gel filtration.

4.9.2. Hemagglutination Studies

Hemagglutination activity was assayed by serial addition technique using albino rat red blood cells (RBC) as described by Lin *et. al.*, (1981). Protein solution (0.2ml) in 5mM phosphate buffer saline, pH-7.2 was mixed with 0.2 ml of 4% (w/v) RBC and the mixture was incubated at 37^0C for an hour. A control containing 0.2 ml PBS instead of protein solution and 0.2 ml of 4% cell suspension was used as reference. The degree of hemagglutination was observed under microscope and the results were recorded as 3^+, 2^+, 1^+ and $\pm$.

The agglutinating activity was expressed as the titer, the reciprocal of the lowest concentration at which visible agglutination could be detected. The specific activity was expressed as the titer per mg of protein.

4.9.3. Hemagglutination Inhibition Studies

The Hemagglutination Inhibition test was performed in the presence of different sugars. Protein solutions (0.1ml) containing minimum concentration of protein needed for visible agglutination were added to 0.1ml sugar solutions of various concentrations and mixed gently. The mixture was then mixed with 0.2 ml of 4% RBC in PBS and incubated at 37^0C for 1 hour. Reactions were compared with a positive control (0.1ml protein solution + 0.1ml PBS + 0.2 ml 4% RBC) and a negative control (0.2ml PBS + 0.2 ml 4% RBC) as reported by Atkinson and Trust (1980).

4.9.4. Protein Concentration

The concentration of protein was measured by the method of Lowry *et. al.*, (1951) using BSA as the standard and the proteins in column elute fractions were also monitored spectrophotometrically at 280 nm.

4.9.5. Analysis of Carbohydrate

The presence of sugar in the purified proteins was detected by periodic acid Schiff's method (Anthony and Andrews, 1978) and percentage of sugar was estimated by phenol- sulfuric acid method (Dubois *et. al.*, 1956) using D-glucose as the standard. For identification of sugars, the protein solutions were hydrolyzed with 4M HCl for 4 hours at 100°C under vacuum. The sugar components of the protein were determined by the one dimensional thin layer chromatography (TLC) method as described by Touchstone and Dobbins (1978) using different standard sugars. The chromatogram was developed with a solvent system of isopropanol, acetic acid and water (3:1:1, v/v/v) in ascending manner and after drying, sprayed with aniline- phthalate solution. The chromato-spots were identified by comparing the R_f values with those of the standard sugars.

4.9.6. Brine Shrimp Lethality

Cytotoxicity was studied using Brine Shrimp eggs. Shrimp eggs were placed in one side of a small tank divided by a net containing sea water (3.8% NaCl solution) for hatching. In the other side of the tank a light source was placed in order to attract the nauplii. Two days were allowed to hatch all the eggs and in this period the nauplii were also sufficiently matured for experiment as depicted by Mayer *et. al.*,(1982).

From the stock solutions of the protein samples, specific volumes were transferred to the different vials containing 10 living shrimps and then sea water was added to make the volume upto 5 ml in each vial. The final concentration of the sample in the vials became 2, 4, 8, 16 and 32 μg/ml respectively. Three experiments were

carried out for the same concentration to get more accurate result and a control experiment was performed similarly taking 10 living shrimps in 5 ml sea water.

After 24 hours incubation, the vials were observed and the number of death in each vial was counted using a magnifying glass. From this data, the mean percentage of mortality of nauplii was calculated at each concentration.

4.9.7. Ultraviolet Absorption Spectra

The ultraviolet absorption spectra of the proteins were recorded in aqueous solution with a Shimadzu Model UV- 180 Double Beam Spectrophotometer at room temperature.

4.10. RESULTS

4.11. Purification of Sajna leaves Proteins

The 100% ammonium sulfate saturated crude protein extract after dialysis against 10 mM Tris-HCl buffer, pH 8.4, was applied to a Sephadex G-50 column at 4°C which was previously equilibrated with the same buffer. As shown in Figure-4.1, the proteins were eluted from the column as one main peak, fraction F-2 and another two small peaks, fractions F-1 and F-3. The active fractions F-2 as indicated by solid bar was pooled, precipitated by ammonium sulfate and purified further by ion exchange chromatography on DEAE- cellulose. The fractions F-1 and F-3 was not used for further study as they contained small amount of protein as well as possessed no significant biological activity.

The ammonium sulfate precipitate of fraction F-2 obtained after centrifugation, was dissolved in distilled water and dialyzed against distilled water for 12 hours and against 10 mM Tris-HCl buffer, pH 8.4 at 4°C for overnight with three changes of buffer. After removal of the insoluble material by centrifugation, the clear supernatant was applied to a DEAE-cellulose column at 4°C, which was pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 and eluted by a linear gradient of NaCl from 0.0 to 0.3 M in the same buffer.

As shown in Figure-4.2 that the column bound proteins were eluted as a single but broad peak, indicating the presence of more than one component in the fraction. In order to separate these components, the elution was carried out stepwisely with increasing concentrations of NaCl in the same buffer.

As shown in Figure-4.3 the components of F-2 fraction were bound tightly to the column and separated into four different fractions, F-2a, F-2b, F-2c and F-2d which were eluted with the same buffer containing 0.06, 0.12, 0.25 and 0.50 M NaCl respectively. It was found that the fractions F-2a, F-2b and F-2c showed biological activities while fraction F-2d was discarded, as it contained no

significant hemagglutination activity. The areas as indicated by solid bar for the active fractions F-2a, F-2b and F-2c were pooled separately and further purified by affinity chromatography on Sepharose-4B.

As shown in Figure-4.4 most of the protein fractions of F-2a, F-2b and F-2c were bound tightly to the affinity matrix and eluted mainly as one main peak by the buffer containing 0.2 M galactose. The purities of the galactose eluted fractions F-$2a_1$, F-$2b_1$ and F-$2c_1$ were checked by SDS- polyacrylamide slab gel electrophoresis.

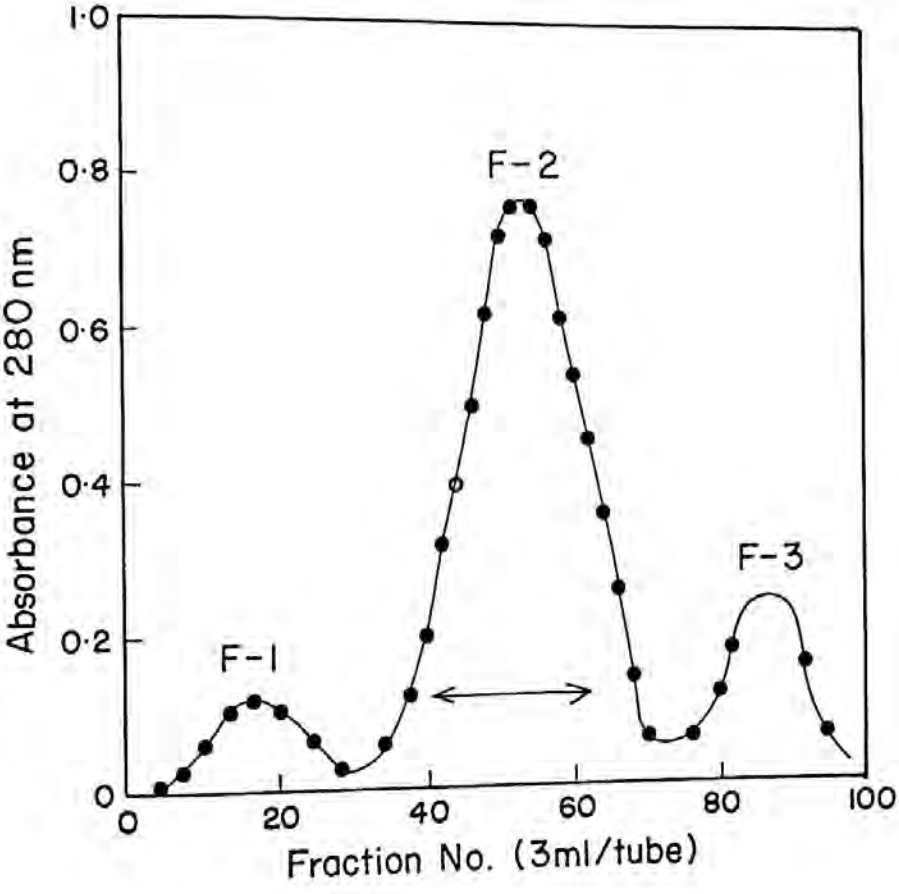

Figure-4.1: Gel filtrarion of 100% $(NH_4)_2SO_4$ saturated crude protein extract on Sephadex G-75. The crude protein extract (30 mg) was applied to the column (2.5x100 cm), pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4°C and developed with the same buffer. Flow rate: 24ml/hour

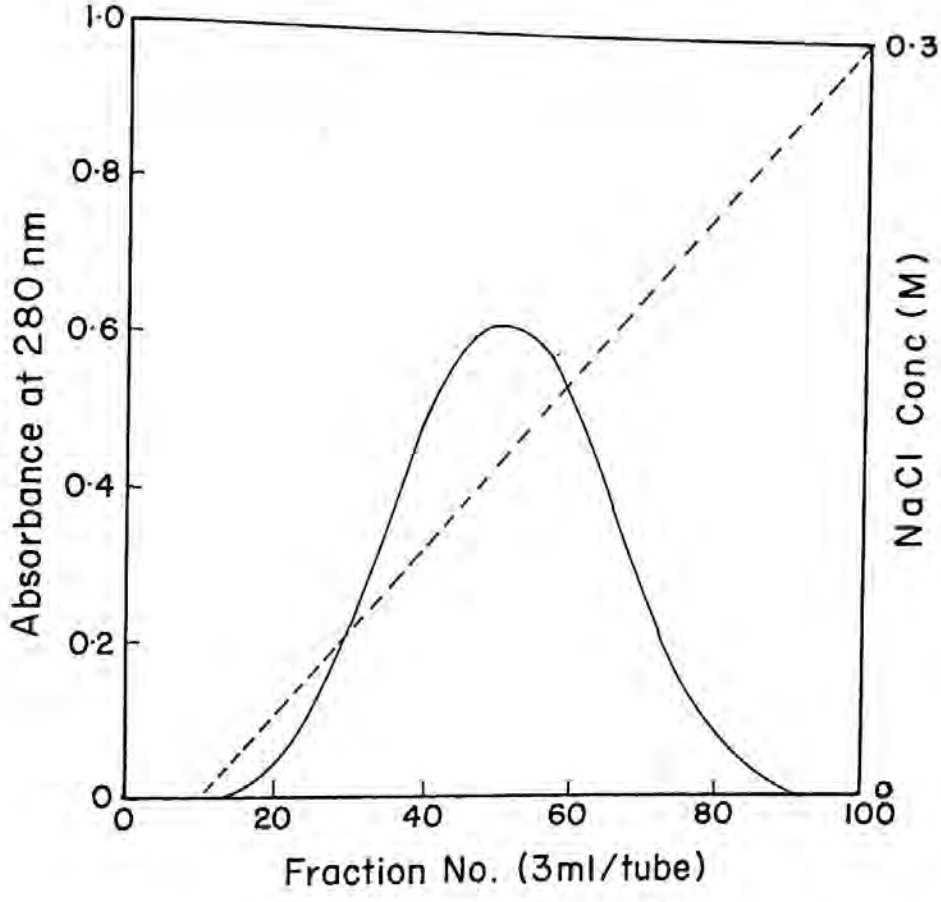

Figure-4.2: Ion exchange chromatography of fraction F-2 on DEAE-cellulose. F-2 (21mg) obtained after gel filtration, was applied to the column (2.1 x 24 cm), which was pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4°C and eluted by a linear gradient of NaCl in the same buffer. Flow rate: 45ml/hour.

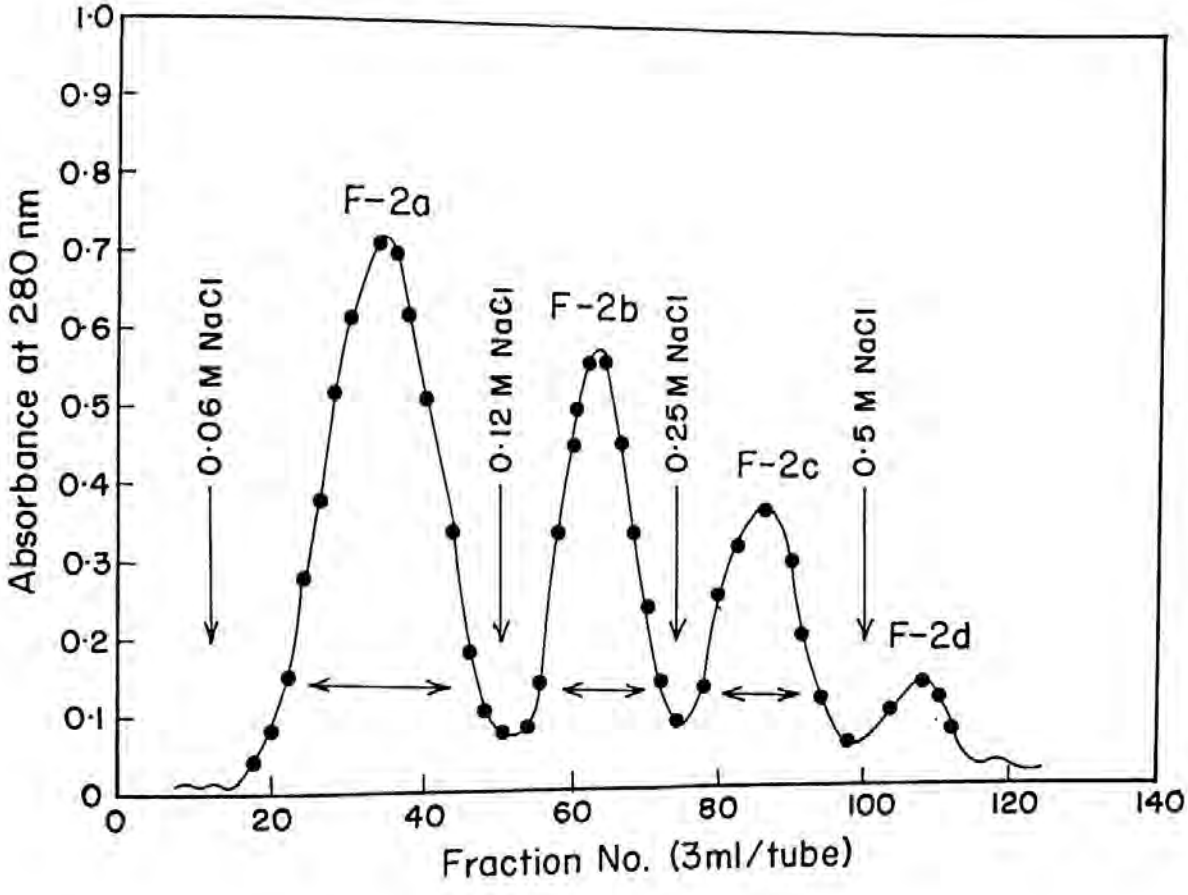

Figure-4.3: Ion exchange chromatography of fraction, F-2 on DEAE-cellulose. F-2 (45 mg), obtained after gel filtration, was applied to the column (21 x 24 cm), pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4°C and eluted by stepwise increases of NaCl concentration in the same buffer. Flow rate: 45ml/hour.

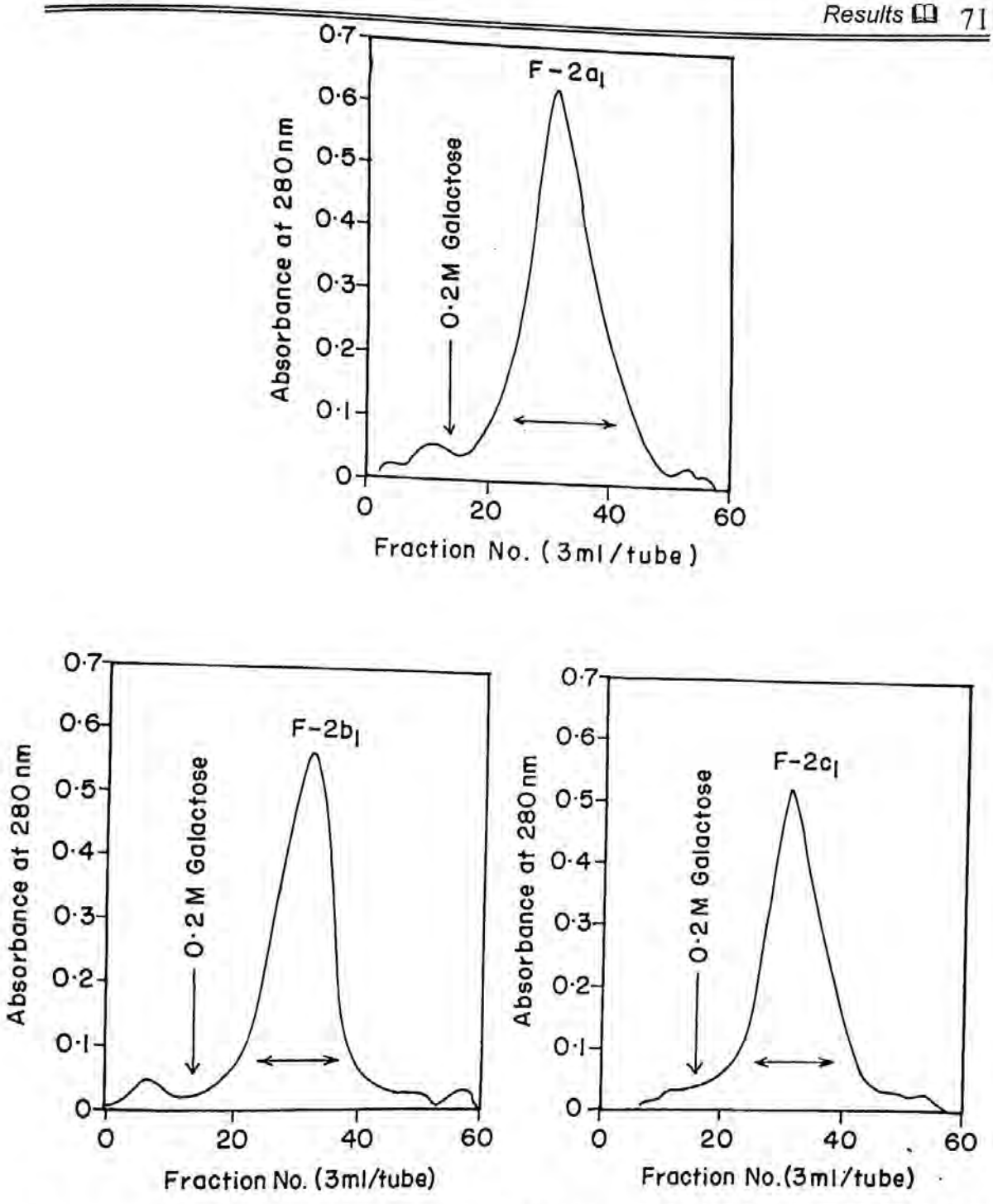

Figure-4.4: Affinity chromatography of fractions, F-2a (10.2 mg), F-2b (8.6 mg) and F-2c (6.8 mg) obtained from DEAE-cellulose column, on Sepharose-4B. The fractions were applied individually to the column (1.25 x 10 cm) pre-equilibrated with 5 mM phosphate buffer saline, pH 7.2 at 4°C and the absorbed proteins were eluted by the same buffer containing 0.2 M galactose. Flow rate: 30ml/hr

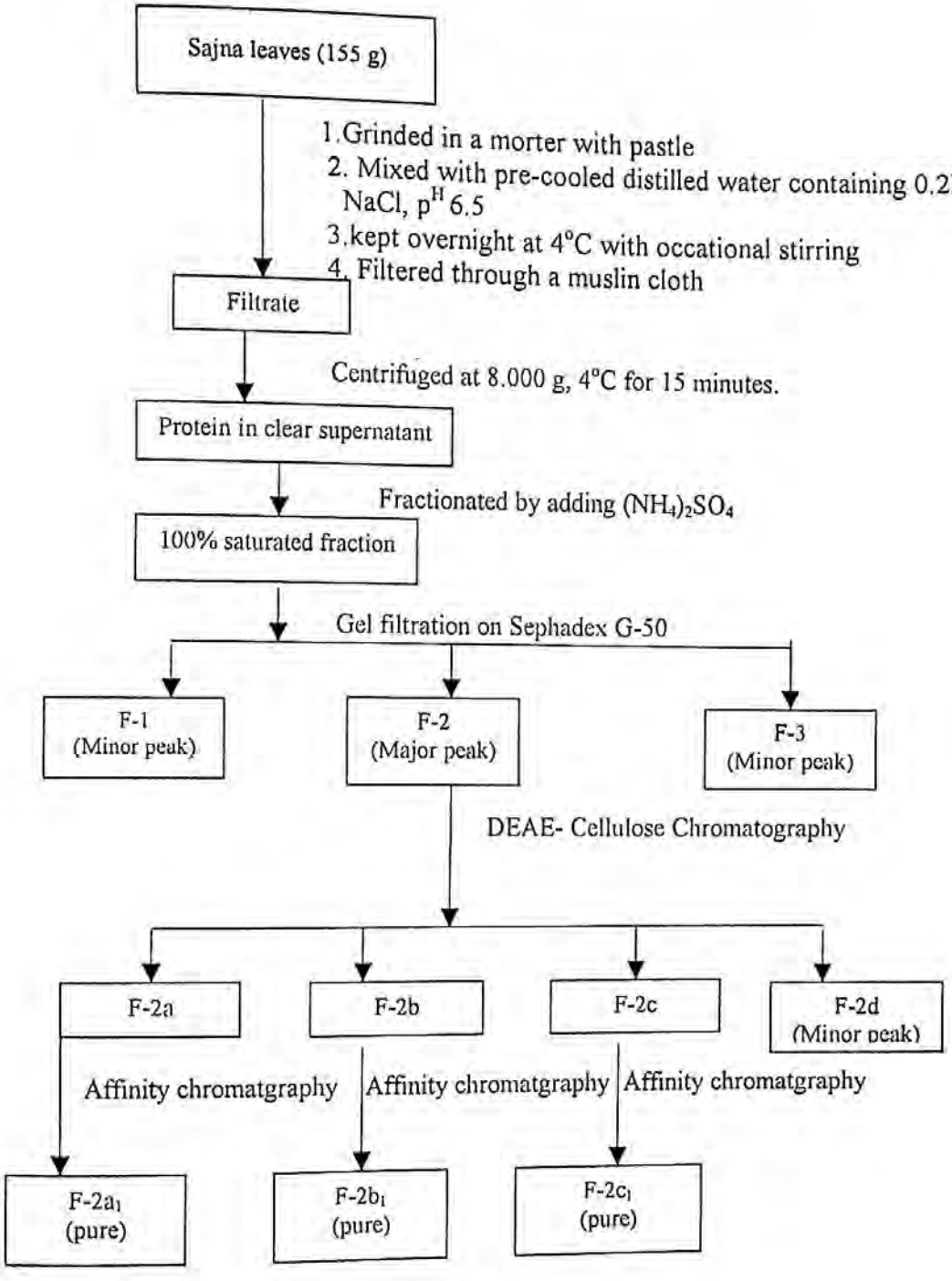

Figure 4.5: Flow diagram for purification of Sajna leaves proteins.

The overall purification data of Sajna leaves proteins were summarized in Table-4.2. From the table, it was evident that the specific activities of the different protein fractions were increased at each purification step and the fraction F-$2a_1$ showed maximum hemagglutination activity with a purification fold of 13.9, while the fraction, F-$2b_1$ and F-$2c_1$ showed the purification fold of 12.1 and 7.17 respectively. Although the yield of these proteins were decreased after each subsequent purification steps and more than 96 % of extracted proteins were destroyed during the purification processes but the purification fold of the proteins were increased after each subsequent purification step. It may suggest from the result that the decrease in yield might be due to the denaturation of proteins during the lengthy purification procedure or some other reasons.

Table 4.2: Purification of Sajna leaves proteins

Fraction		Total protein (mg)	Hemagglutination activity (titre)	Specific activity (titre/mg)	Yield (%)	Purification fold
Crude extract		550	3210	5.06	100	1.00
100% $(NH_4)_2SO_4$ saturated fraction		200	2230	9.72	69.47	1.92
After gel filtration		82	1740	16.28	54.20	3.21
DEAE-cellulose fractions	F-2a	10.2	560	54.9	24.56	10.8
	F-2b	8.6	410	47.6	17.98	9.40
	F-2c	6.8	215	31.6	9.42	6.24
Affinity chromatography	F-$2a_1$	7.1	500	70.4	21.92	13.9
	F-$2b_1$	6.0	370	61.66	16.66	12.1
	F-$2c_1$	5.2	189	36.3	8.28	7.17

Hemagglutination (Hg) activity (titre) = Reciprocal of lowest concentration showing visible hemagglutination.

$$\text{Yield} = \frac{\text{Observed Hg-activity}}{\text{Initial Hg-activity}} \times 100$$

$$\text{Fold} = \frac{\text{Observed Specific activity}}{\text{Initial specific activity}}$$

4.12. Molecular Weight of the Proteins

a) Gel Filtration

The molecular weight of the purified Sajna leaves proteins were determined by gel filtration on Sephadex G-150 column using β-amylase, β-D-galactosidase, bovine serum albumin, egg albumin and lysozyme, as marker proteins under identical experimental conditions. The molecular weight of the purified proteins were calculated from the standard curve, which was constructed by plotting molecular weight against elution volume of the marker proteins and were found to be about 1,55,000; 1,15,000 and 85,000 for F-$2a_1$, F-$2b_1$ and F-$2c_1$ respectively. (Figure 4.6)

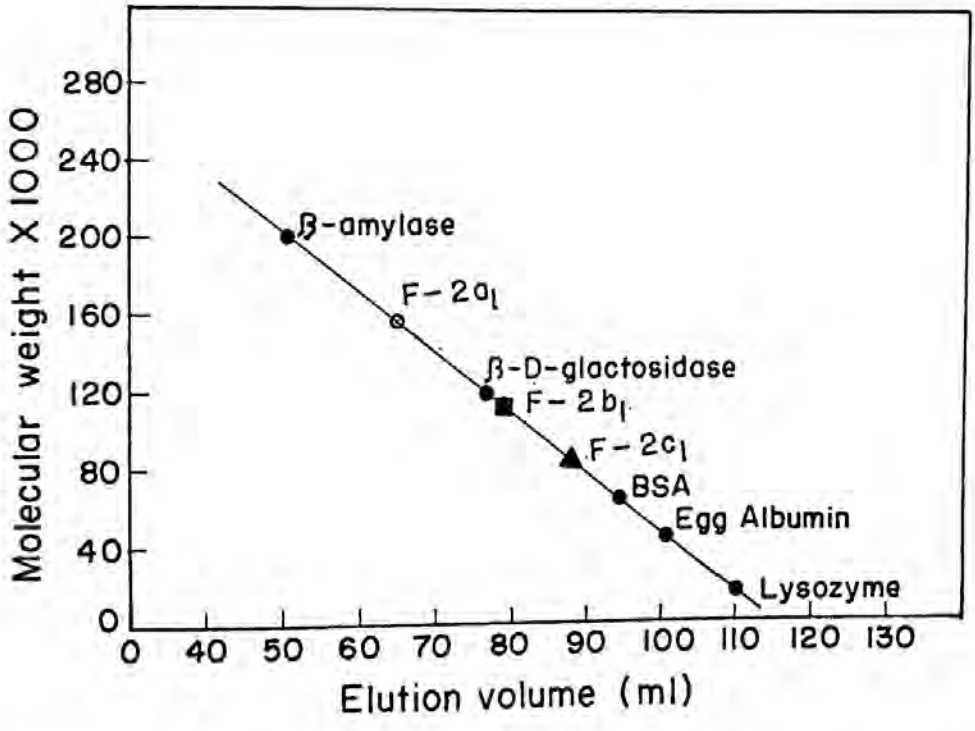

Figure 4.6: Standard curve for determination of molecular weight by gel filtration on Sephadex G-150. Proteins were applied to a Sephadex G-150 column (1.6 x 90 cm) pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4°C and eluted with the same buffer. Flow rate: 15ml/hour.

b) **SDS-PAGE**

As shown in **Fig.4.6** all the three fractions obtained after affinity chromatography on Sepharose-4B gave single band in the gel which was performed in the presence of 10% SDS-polyacrylamide slab gel electrophoresis, indicating that all the three fractions contained pure protein.

The molecular weight of the proteins was calculated by comparing the distances moved by the standard proteins with those of the proteins on the SDS-polyacrylamide slab gel (**Fig. 4.7**) and the molecular weight of **F-2a$_1$**, **F-2b$_1$** and **F-2c$_1$** were estimated to be 1,60,000; 1,20,000 and 85,500 respectively (**Fig. 4.8**). The MW of *vicia unijuga* leaves anti-N lectin by SDS-PAGE was reported to be 120,000 (Khomei Yanagi *et. al.*, 1990) and the MW of mulberry seeds lectins were 1,75,000; 1,20,000 and 89,500 for MSL-1, MSL-2 and MSL-3 respectively (Tanzima Yeasmin *et. al.*, 2001). Further, the subunit structure of three proteins were determined by SDS-electrophoresis in the presence of SDS and β-mercaptoethanol (Fig. 4.9) F-2a$_1$ gave a strong band corresponding to a molecular mase of 1,00,000 and a slightly faint band corresponding to a molecular mase of 60,000 and F-2b$_1$ gave two bands corresponding to a molecular mase of 65,000 and 55,000 while F-2c$_1$ gave single band with the molecular weight of 85,500 (Fig. 4.10). The MW of *vicia unijuga* leaves anti-N lectin by SDS-PAGE was reported to be 120,000 and it is monomer in nature (Khomei Yanagi *et. al.*, 1990).

Fig. 4.7: Photographic representation of purified proteins on 10% SDS-polyacrylamide slab gel electrophoresis.

Staining reagent : Coomassic brilliant blue

Protein concentration : 75μg

Lane A = Standard markers; Lane B = F-2a_1;

Lane C = F-2b_1; Lane D = F-2c_1;

Lane E = Crude protein.

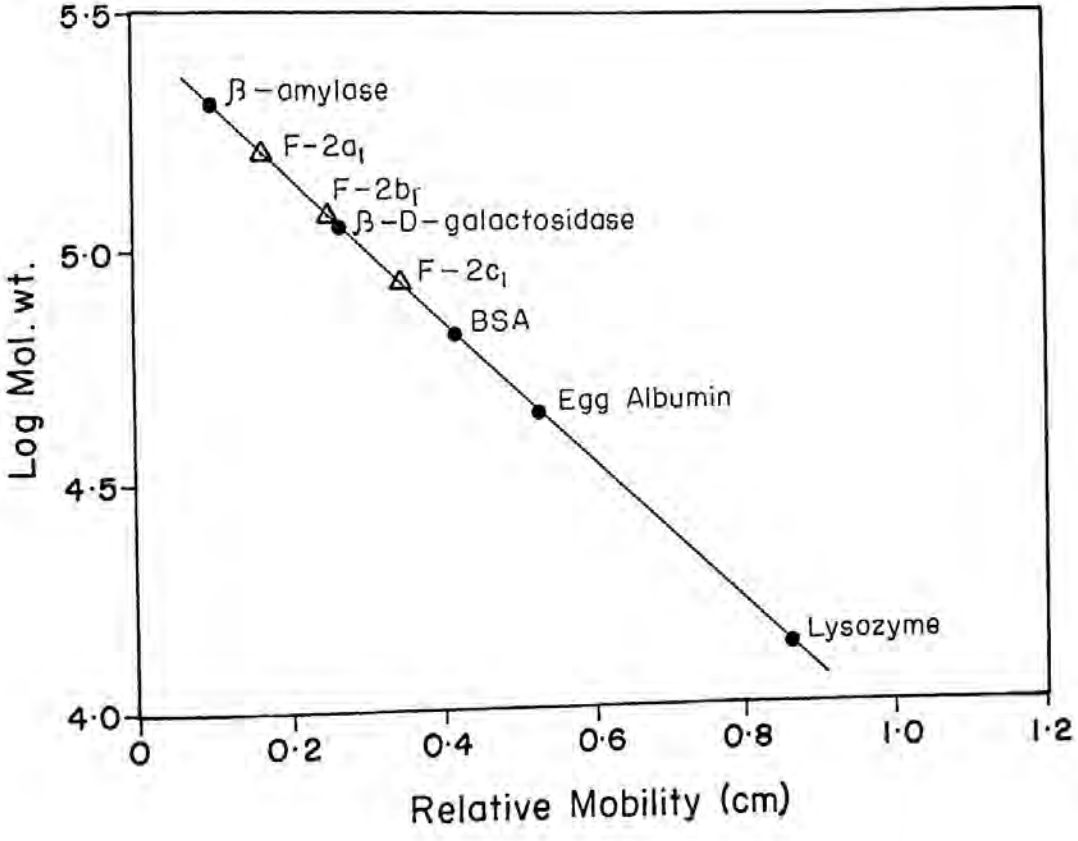

Fig. 4.8: Determination of molecular weight of purified proteins by SDS-slab gel electrophoresis.

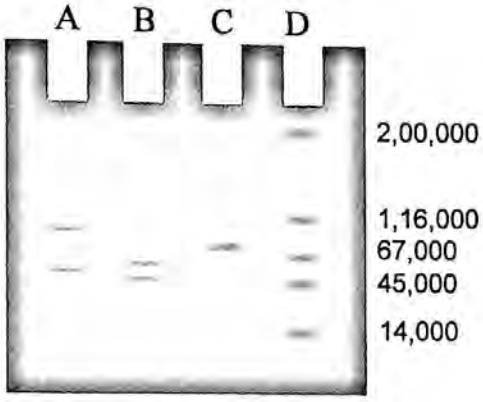

Fig. 4.9: SDS-polyacrylamide slab gel electrophoretic pattern of the proteins at room temperature.

Staining reagent : 0.1% Coomassie brilliant blue R-250,

Protein concentration : 75μg

Lane A = F-2a_1; Lane B = F-2b_1; Lane C = F-2c_1;

(In presence of β-mercaptoethanol)

Lane D = Standard markers

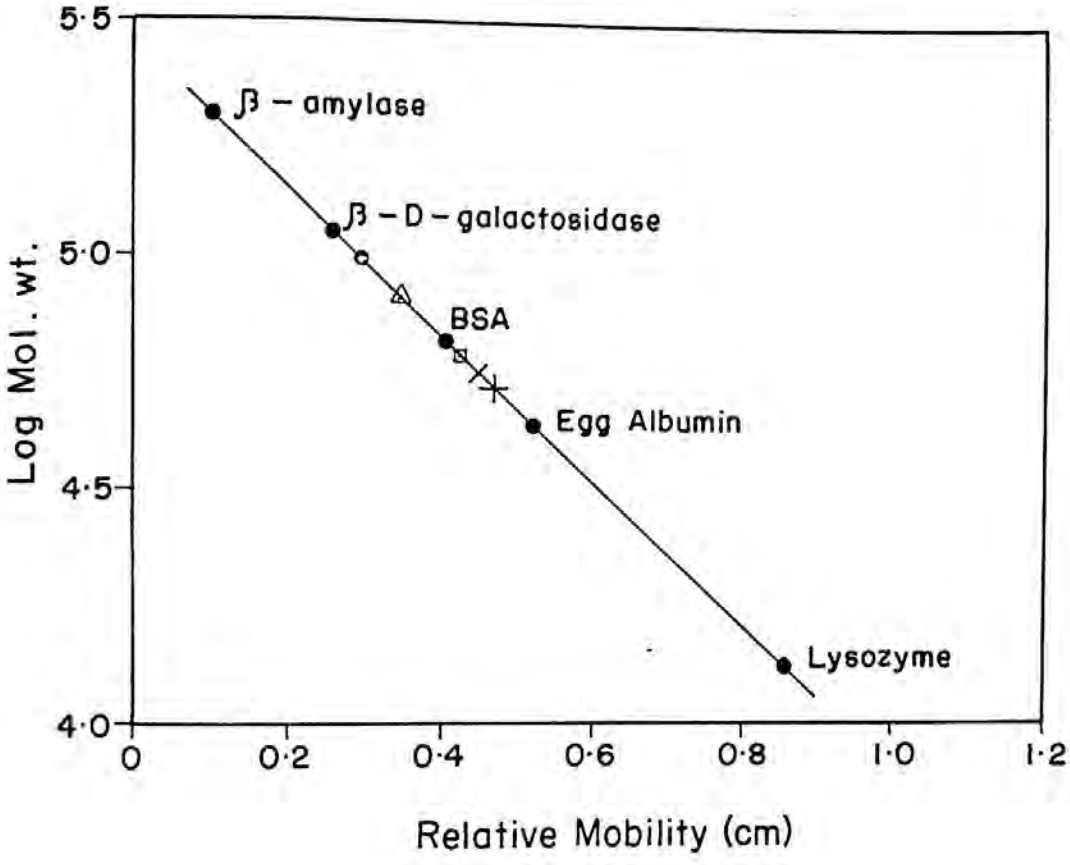

Fig. 4.10 Determination of Molecular weight of purified protein's subunit by SDS-slab gel electrophoresis.

- o F-2a_1 (MW 1,00,000)
- — F-2a_1 (MW 60,000)
- ▫ F-2b_1 (MW 65,000)
- \+ F-2b_1 (MW 55,000)
- △ F-2c_1 (MW 85,500)

4.12.1. Hemagglutinating Activities

All the proteins agglutinated specifically the albino rat red blood cells (RBC). Table- 4.3 shows the degree of hemagglutination of albino rat red blood cells by F-2a_1, F-2b_1 and F-2c_1 and the photographic representations of hemagglutination are shown in figure 4.11, 4.12 and 4.13 respectively. A minimum protein concentration of 4.4, 6.9 and 11.5 μg/ml of F-2a_1, F-2b_1 and F-2c_1 respectively were needed for visible agglutination (Table- 4.3). From these observations, it can be concluded that the purified proteins are lectin in nature and the proteins F-2a_1, F-2b_1 and F-2c_1 are denoted as SLL-1, SLL-2 and SLL-3 respectively.

Table- 4.3: Hemagglutinating activities of Sajna leaves proteins with 4% red blood cells (RBC) from albino rat.

Protein samples	Absorbance at 280 nm	Conc. of protein (μg/ml)	Degree of hemagglutination
SLL-1	0.0720	11.5	3^+
	0.050	9.2	2^+
	0.0320	6.9	1^+
	0.0100	4.4	±
SLL-2	0.0850	20.0	3^+
	0.070	16.8	2^+
	0.0550	12.3	1^+
	0.038	6.9	±
SLL-3	0.0902	24.2	3^+
	0.0812	19.3	2^+
	0.0780	16.5	1^+
	0.0542	11.5	±

3^+ Indicates complete aggregation of almost all the cells.

2^+ Indicates lesser degree of agglutination where smaller number of cells remained free.

1^+ Indicates all the cells were present in small aggregation of varying sizes.

± Indicates major cells were present in aggregates.

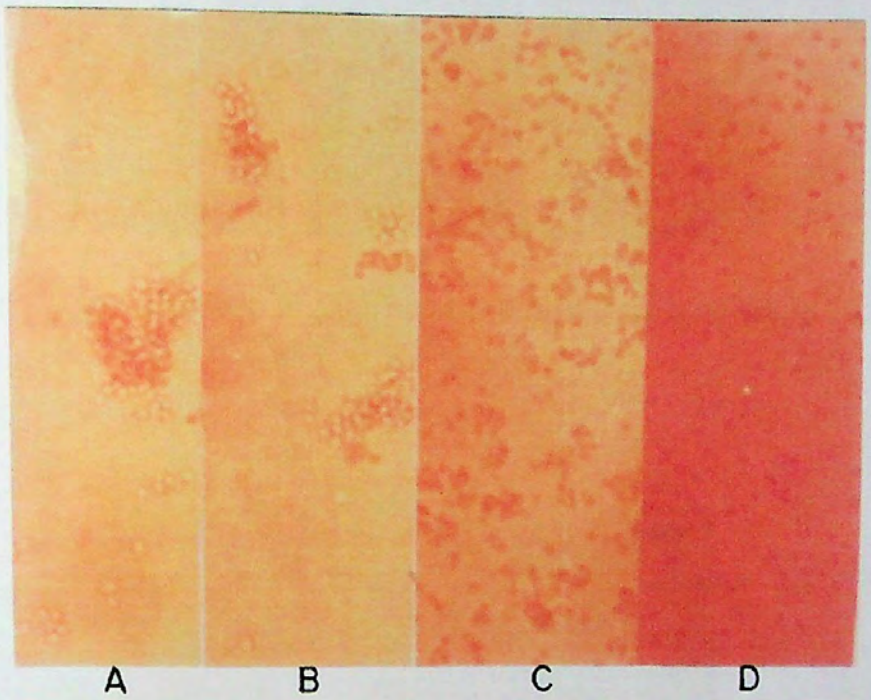

Fig. 4.11: Agglutination of albino rat red blood cells by SLL-1
A = 3^+; B = 2^+; C = 1^+ and D = control

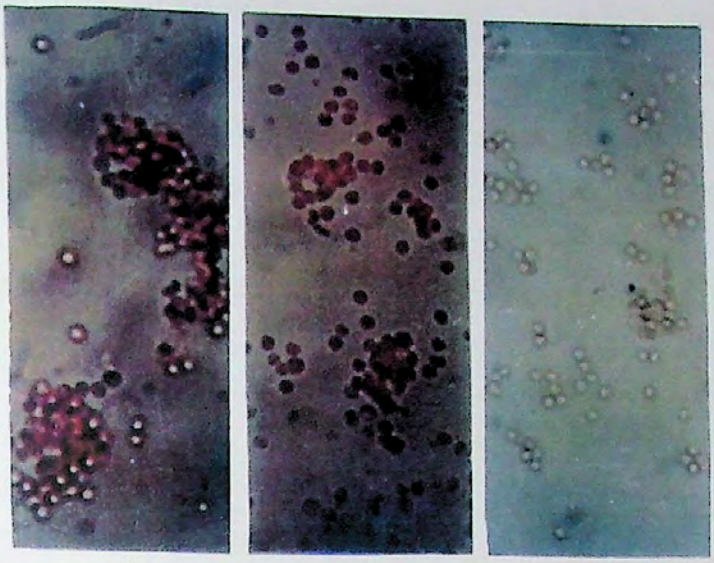

Fig. 4.12: Agglutination of albino rat red blood cells by SLL-2
A = 3^+; B = 2^+ and C = 1^+

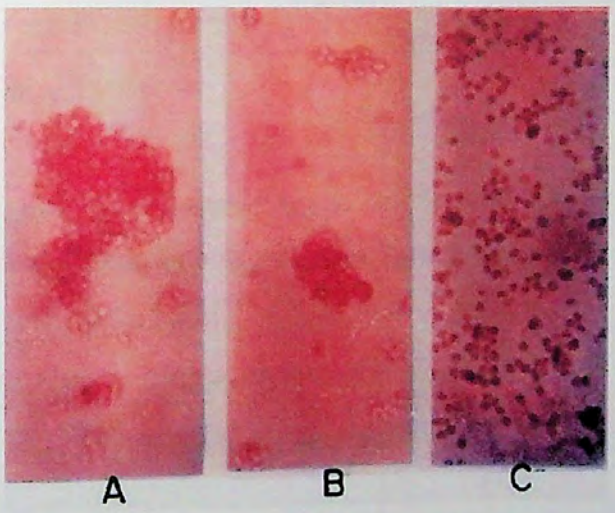

Fig. 4.13: Agglutination of albino rat red blood cells by SLL-3
A = 3^+; B = 2^+ and C = 1^+

3^+. Indicates complete aggregation of most of the cells.
2^+. Indicates lesser degree of agglutination where smaller number of cells remained free.
1^+. Indicates all cells were present in small aggregation of varying sizes.

4.12.2. Hemagglutination Inhibition Studies

Hemagglutination Inhibitions of the lectins SLL-1, SLL-2 and SLL-3 were performed in the presence of different sugars and the results were depicted in Table-4.4. Galactose and galactose containing saccharides are found to be highly effective for inhibiting the agglutination of rat red blood cells by all the lectins.

Table-4.4: Hemagglutination Inhibition assay of Sajna leaves lectins by different sugars.

Proteins	Sugars	Concentration (nM)	Hemagglutination
SLL-1	D-Glucose	100	NI
	D-Mannose	100	NI
	Methyl-α-D galactopyranoside	25	I
	Methyl-β-D galactopyranoside	25	I
	D-Galactose	20	I
	D-glucosamine-hydrochloride	110	NI
SLL-2	D-Glucose	110	NI
	D-Mannose	85	NI
	Methyl-α-D galactopyranoside	25	I
	Methyl-β-D galactopyranoside	25	I
	D-Galactose	20	I
	D-glucosamine-hydrochloride	110	NI
SLL-3	D-Glucose	80	NI
	D-Mannose	100	NI
	Methyl-α-D galactopyranoside	30	I
	Methyl-β-D galactopyranoside	25	I
	D-Galactose	20	I
	D-glucosamine-hydrochloride	100	NI

I = Inhibition, NI = No Inhibition

4.12.3. Protein Concentration

The absorbance of 1.0 at 280 nm for SLL-1, SLL-2 and SLL-3 were found to be equal to 0.68, 0.73 and 0.83 mg of proteins, respectively as determined the concentration of protein by the Lowry method (Table-4.5).

Table-4.5: Optical density (O.D.) and concentration relationship of the proteins.

Proteins	O.D. of proteins at 280 nm	Amount of proteins (mg)
SLL-1	1.0	0.68
SLL-2	1.0	0.73
SLL-3	1.0	0.83

4.12.4. Analysis of Carbohydrate

The presence of sugars in the proteins were confirmed by periodic acid schiff's (PAS) staining method (Khomei Yanagi *et al.*, 1990). It was found that all the proteins produced pinkish red band on polycarylamide gel, when the gels were stained with PAS reagent after electrophoresis (Fig. 4.14). The neutral sugar contents as determined by phenol-sulphuric acid method of the lectins SLL-1, SLL-2 and SLL-3 were estimated to be 3.9, 3.4 and 2.8% respectively. The sugar composition of the lectins as identified by one dimensional thin layer chromatography (TLC) was found to be glucose for SLL-1 and mannose for SLL-2 while SLL-3 may contain either N- acetyl-D-glucosamine or N- acetyl-D-galactosamine or both (Table-4.6).

Fig. 4.14: Polyacrylamide slab gel electrophoretic patterns of purified protein on 7.5% gel after staining with periodic acid schiff's (PAS) reagent. Lane A = SLL-1; Lane B = SLL-2 and Lane C = SLL-3.

Table 4.6: Data of the R_f values obtained by TLC examination of the sugars obtained from hydrolyzed protein samples and standard sugars.

Standard sugars & protein sample	Distance traveled by the solvent system (cm)	Distance traveled by the standard sugar and protein (cm)	R_f values
Sugar:			
D-Glucose	13.9	9.34	0.672
D-Galactose	13.9	10.44	0.751
D-Mannose	13.9	8.80	0.633
D-Arabinose	13.9	11.02	0.792
N-acetyl-D-glucosamine	13.9	11.46	0.831
N-acetyl-D-galactosamine	13.9	11.71	0.842
Protein:			
SLL-1	13.9	9.20	0.662
SLL-2	13.9	9.0	0.647
SLL-3	13.9	11.22	0.839

4.12.5. Cytotoxic effects

All the three lectins exhibited significant toxic effects against brine shrimp lethality bioassay. The mortality rate of brine shrimp nauplii was found to increase with the increase in concentration of the lectins and a plot of log of concentration vs. percent of mortality gave almost linear correlation (Figure-4.15).

From the Figure-4.15, the LC_{50} (concentration at which 50% mortality of the nauplii occurs) as estimated by the extrapolation was found to be 15.8 μg/ml for SLL-1, 17.78 μg/ml for SLL-2 and 14.12 μg/ml for SLL-3 (Table-4.6).

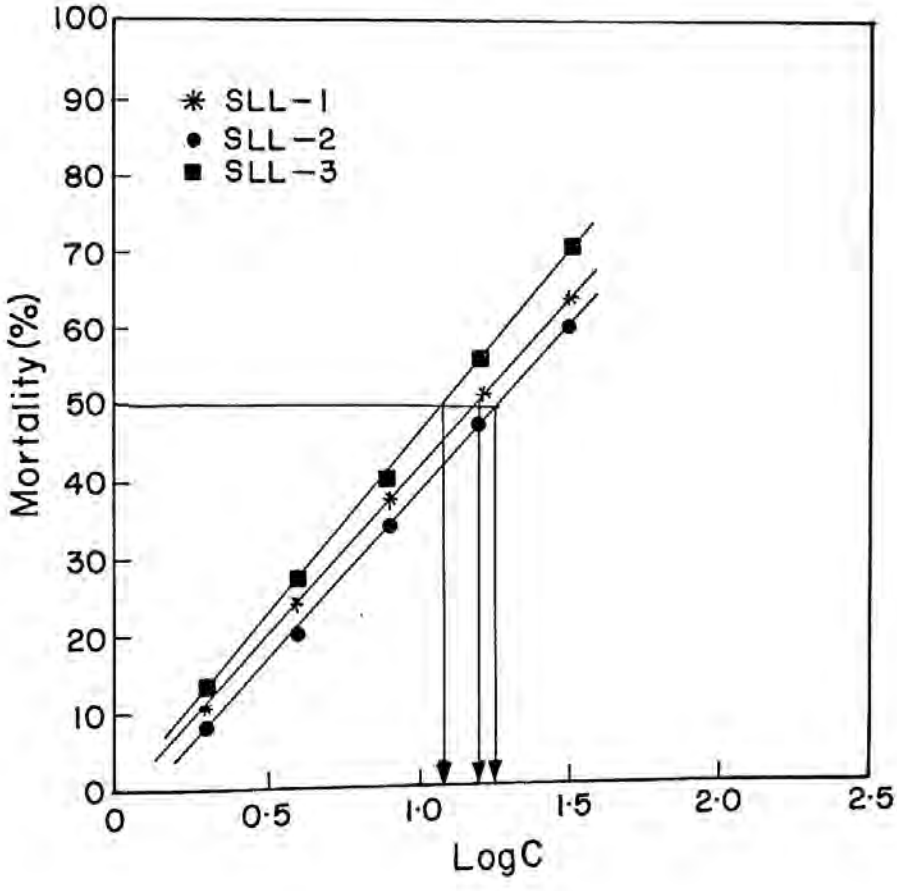

Figure-4.15: Determination of LC_{50} of Sajna leaves lectin.

Table-4.7: Effect of Sajna leaves lectins on brine shrimp lethality bioassay.

Test sample	Conc. (µg/ml)	Log conc. (LogC)	No.of shrimp taken	No. of survival (Average)	Mortality (%)	LC_{50} (µg/ml)
Control	0	0	10	0	0	
SLL-1	2	0.3010	10	88	12	15.8
	4	0.6020	10	76	24	
	8	0.9030	10	62	38	
	16	1.2041	10	49	51	
	32	1.5051	10	36	64	
SLL-2	2	0.3010	10	92	8	17.78
	4	0.6020	10	79	21	
	8	0.9030	10	66	34	
	16	1.2041	10	52	48	
	32	1.5051	10	40	40	
SLL-3	2	0.3010	10	87	13	14.12
	4	0.6020	10	72	28	
	8	0.9030	10	59	41	
	16	1.2041	10	44	56	
	32	1.5051	10	29	71	

4.12.6. Ultraviolet Absorption Spectra

The ultraviolet absorption spectra of the lectins were recorded in aqueous solution with a Shimadzu Model UV-180 Double Beam Spectrophotometer at room temperature. The purified lectins SLL-1, SLL-2 and SLL-3 in aqueous solution gave absorption maximum around 273, 275, and 278 nm and minimum around 251, 253 and 258 nm respectively (Figure-4.16).

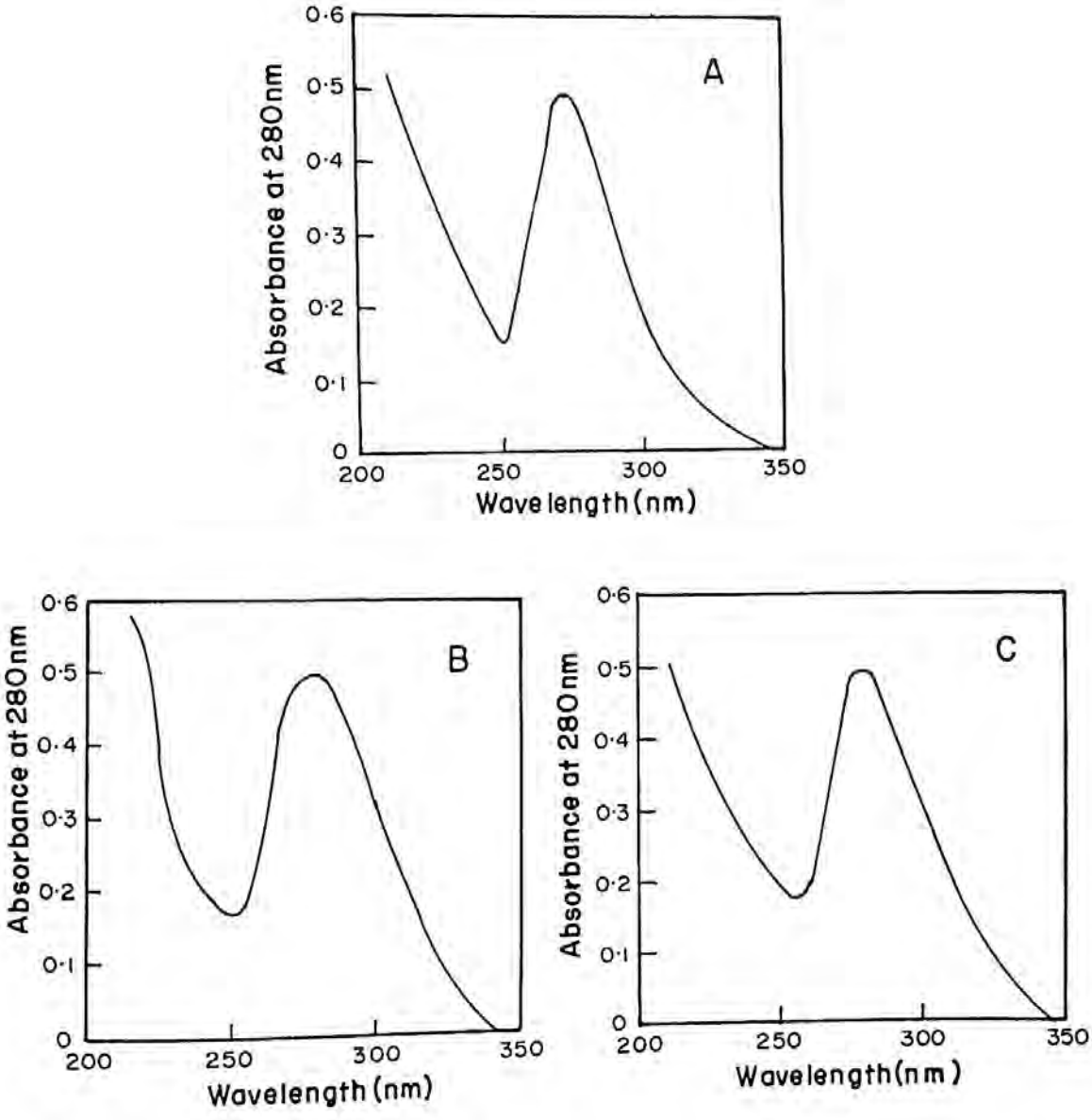

Figure-4.16: Ultraviolet absorption spectra of SLL-1, SLL-2 and SLL-3, lectins in aqueous solution.

A= SLL-1, B= SLL-2 and C= SLL-3

4.13. DISCUSSION

Three proteins have been isolated and purified from the crude protein extract of the Sajna leaves with a purification fold of 33.17 as compared to that of the crude protein extract. All these proteins are glycoproteins in nature as they gave orange yellow color in the presence of phenol-sulfuric acid. The presence of sugar in the lectins was further confirmed by the pinkish red band produced on SDS-polyacrylamide slab gel when stained with periodic acid Schiff's staining reagent after electrophoresis.

The agglutinations of rat red blood cells by the proteins are inhibited specifically in the presence of galactose and galactose containing saccharide. This findings were further supported by the facts that all the three lectins showed binding affinity to Sepharose 4B during purification processes. It is concluded from these above findings that Sajna leaves contained at least three lectins that are specific for D-galactose.

Although the purified lectins from Sajna leaves are similar in their sugar specificities, but quite differ from each other in their molecular weight, neutral sugar content and sugar composition. All the three lectins moved as a single band with distinct identible difference mobilities on the gel after SDS-polyacrylamide slab gel electrophoresis in the presencs of 10% gel and the molecular weights of the lectins SLL-1, SLL-2 and SLL-3 were found to be about 1,55,000; 1,15,000 and 85,000 respectively by gel filtration and 1,60,000; 1,20,000 and 85,500 respectively by SDS-PAGE. Further in the presence of SDS and β-mercaptoethanol, SLL-1 and SLL-2 gave two bands on the gel indicating that both the lectins are dimer in nature which are held together by disulfide bonds. On the other hand, SLL-3 gave single band on the gel indicating that SLL-3 is monomer. A lectin purified from *Bauhinia*

pentandra seeds also contained one subunit with molecular weight of 30,000 (Silva *et al.*, 2001). Again, it may suggest from the results that the monomeric lectin, SLL-3 may contain two binding sites on the same subunit for showing the properties of lectin. Further study is needed to confirm more precisely the saccharide binding site(s) of SLL-3. The lectins purified from plant sources contained four subunits of two identical pairs viz, Indian bean (*Dolichos lablab* L.) (Guruan *et. al.*, 1983), *Arbus precatorius* (Absar and Funatsu, 1984) and Ricinus comminis agglutinin (Olsnes *et. al.*, 1974) but some lectins are dimer with the two monomers held together by disulfide bond (Tanzima Yeasmin *et. al.*, 2001; Marilynn E. Etzler, 1994) while *Vicia unijuga* leaves anti-N lectin is monomer (Khomei Yanagi *et. al.*, 1990)

Interestingly, like other plant lectins, lectins from Sajna leaves are also cytotoxic in nature as they affect significantly the mortality rate of brine shrimp.

In conclusion, the purified lectins SLL-1, SLL-2 and SLL-3, besides being specific for rat red blood cells agglutination, can be added as an addition of members to the list of the purified member of galactose specific lectins. Among the lectins purified, SLL-3 is found to be slightly more toxic followed by SLL-1 and then SLL-2.

CHAPTER-5

Effect of Physico-chemical Agents on the Hemagglutinating Activities of Sajna leaves Lectins.

5.1. Introduction

The conformation of a native or highly organized protein reflects a delicate balance among a variety of interaction forces both within the folded protein interiors and with surrounding solvent. If the protein's solvent environment is perturbed the protein's native conformation can be disrupted, with a resulting loss of function and the production of partially unfolded or denatured protein. The conditions giving rise to partial denaturation and consequent loss of function may be subtle such as small change in pH , temperature and ionic strength on dielectric constant of the medium. Conversely, prolonged boiling or exposure to thiol containing compounds and denaturants such as sodium dodecyl sulfate or to hydrogen bond- breaking reagents such as urea or guanidine hydrochloride may be required for complete denaturation (Zubey, 1988).

The three dimentional structure of a protein is governed by its primary structure and its environment. The organized native structure (conformation) of a protein is known to be affected from the effect of external environmental changes such as temperature, acidity, urea or denaturant solutions and a number of other chemicals. In structural studies of proteins, it is often necessary to established conditions for reversible denaturation. The choice of denaturation condition depends on the stability of the protein of interest. Among the techniques used for reversible denaturation are lowering of the pH (Itano & Singer, 1958), freezing and thawing in concentration of salt (Marker, 1963) and adding denaturants such as urea and guanidine-HCl (Chilson *et. al.*,1964 & 1965, Meighen and Schachman, 1970a & 1970b).

Moringa oleifera L. leaves lectins are glycoproteins in nature. All the three proteins, purified from its leaves specifically agglutinate rat red blood cells and may also be used to perform many other biological activities. In the present study, the lectins have been subjected to various physical and chemical treatments, and their effects on the hemagglutination activities were analyzed. The study is expected to provide important information regarding some of the physico-chemical properties such as pH stability, thermal stability and the stability of the lectins towards denaturing agents.

5.2. MATERIALS AND METHODS.

5.3. Chemicals

Acetic acid and urea were the products of British Drug House (BDH), poole, England. Guanidine-HCl was the product of Bio-Rad Laboratories, Rich mond, California, USA. All other reagents used were of analytical grade.

5.4. Hemagglutinating Activity

After various physico-chemical treatments of protein solutions as depicted below, the hemagglutinating activity was determined according to the method of *Lin et. al.*, (1981) using 4% albino rat red blood cells, as described in Chapter 2.

5.5. Physical treatments of the lectins

i) Effect of pH

Lectin solutions in 50 mM of respective buffers possessing pH ranges from 2.0-10.5 were incubated for 10 hours at 28 °C. The hemagglutinating activity retained was determined after dialysis of the lectin solutions against 5mM phosphate buffer saline pH 7.2 for 18 hours at 4°C.

ii) Effect of Temperature

Lectin solutions in 5 mM phosphate buffer saline pH 7.2 were heated at various temperature for 1 hour using a temperature controlled water bath. After cooling the heated lectin solution in an ice bath, the hemagglutinating activity was determined.

5.6. Chemical Treatments of the lectins

i) Treatment with Acetic Acid

Lectin solutions (200µl) in 5 mM phosphatr buffer saline pH 7.2 were mixed with acetic acid at different concentrations. After an incubation period of 1 hour at 4°C,

the lectin solutions were dialyzed against 5mM phosphate buffer saline pH 7.2 for 12 hours at 4°C and then hemagglutinating activity was determined.

ii) Treatment with Urea

Solid urea of different concentrations was added to the lectins in 10 mM Tris-HCl buffer, pH 8.2. The solutions were incubated at 15°C for 12 hours and then dialysed against 5mM phosphate buffer saline pH 7.2 for overnight at 4°C to remove urea. The hemagglutinating activity of the dialyzed solution was determined.

iii) Treatment with Guanidine - HCl

To the lectin solutions in 10 mM Tris-HCl buffer, pH 8.2 was added solid Guanidine - HCl of different concentrations. After incubation at 20°C for 12 hours, the solutions were dialyzed against 5mM phosphate buffer saline pH 7.2 for 12 hours at 4°C. The hemagglutinating activity of the dialyzed solution was determined.

iv) Treatment with various metalic salts

The metal salts of different concentrations (in 5mM phosphate buffer, pH-7.2) were added to the lectin solutions (200 μl) and incubated for 30 min. at room temperature. The hemagglutinating activity was assayed after incubating at 37°C for 1 hour. In this experimental procedure deionized water was used.

5.7. RESULTS

i) Effect of pH on hemagglutinating activities of the lectins.

The effect of pH on hemagglutinating activities of SLL-1, SLL-2 and SLL-3 are shown in Table 5.1. Results indicated that the biological activities of the three lectins were remarkably influenced by the pH changes. The hemagglutinating activities of the lectins were found to be much higher in mild basic pH region than the acidic pH values and the maximum activities were observed between pH 7.2 to 7.6. Beyond these pH values the activities of the lectins decreased at the acidic as well as basic pH regions and the lectins lost their activities almost completely around pH 2.0 and pH 10.5.

Table-5.1: Hemagglutinating activities of Sajna leaves lectins at different pH values.

Buffer composition	pH	Relative hemagglutinating activities (%)		
		SLL-1	SLL-2	SLL-3
KCl-HCl	2.0	0.0	5.0	0.0
AcONa- HCl	3.0	5.0	10	5
AcONa-CH_3COOH	4.0	15	20	20
AcONa-CH_3COOH	5.0	25	30	40
NaH_2PO_4- Na_2HPO_4	6.0	50	50	65
NaH_2PO_4- Na_2HPO_4	6.5	70	75	80
NaH_2PO_4- Na_2HPO_4	7.2	100	100	100
NaH_2PO_4- Na_2HPO_4	7.6	90	100	95
NaH_2PO_4- Na_2HPO_4	8.0	70	90	80
$Na_2B_4O_7$-HCl	9.5	30	40	25
$Na_2B_4O_7$-Na_2CO_3	10.5	5	00	5

ii) Effect of temperature on hemagglutinating activities of the lectins.

The hemagglutinating activities of SLL-1, SLL-2 and SLL-3 were also affected remarkably with the changes of temperature. As mentioned in Table 5.2 the lectins gave maximum hemagglutinating activities around the temperature 20-35°C. Again, the hemagglutinating activities of the lectins decreased rapidly with further rise of temperature and the activities were destroyed completely at or above 75°C.

Table-5.2: Effect of temperature on hemagglutinating activities of Sajna leaves lectins.

Temperature (°C)	Relative hemagglutinating activities (%)		
	SLL-1	SLL-2	SLL-3
20	100	100	100
25	100	100	100
30	100	100	100
35	100	95	100
40	85	90	85
50	55	65	70
60	20	35	30
70	15	20	15
75	0	0	0

iii) Effect of acetic acid on hemagglutinating activities of the lectins.

As shown in Table-5.3, the hemagglutinating activities of SLL-1, SLL-2 and SLL-3 were fully retained even after treatment with 0.5% acetic acid and were then decreased sequentially with further increase in acetic acid concentration and more than 75% activities of SLL-1 and SLL-2 were lost at 20% acetic acid concentration while SLL-3 lost its activity completely at 20% acetic acid concentration.

Table-5.3: Effect of acetic acid at different concentrations on hemagglutinating activities of Sajna leaves lectins.

Concentration of acetic acid (%)	Relative hemagglutinating activities (%)		
	SLL-1	SLL-2	SLL-3
0.0	100	100	100
0.5	100	100	100
2.5	95	90	80
5.0	70	75	50
10	40	50	40
20	20	25	00
30	00	00	00

iv) Effect of urea on hemagglutinating activities of the lectins.

The hemagglutinating activities of SLL-1, SLL-2 and SLL-3 were decreased sequentially with increased in urea concentration and the activities were abolished completely after treatment with 9M urea (Table-5.4). The results also indicated that SLL-3 is slightly more sensitive to urea than those of SLL-1 and SLL-2.

Table-5.4: Effect of urea at different concentrations on hemagglutinating activities of Sajna leaves lectins.

Concentration of urea (mole)	Relative hemagglutinating activities (%)		
	SLL-1	SLL-2	SLL-3
0	100	100	100
1	100	100	100
2	85	90	75
4	65	70	60
6	40	45	35
8	20	15	10
9	00	00	00

v) Effect of Guanidine-HCl on hemagglutinating activities of the lectins.

The hemagglutinating activities of SLL-1, SLL-2 and SLL-3 were affected markedly after treatment with guanidine-hydrochloride. The results as presented in Table-5.5 indicated that SLL-1, SLL-2 and SLL-3 retained only 10%, 25% and 15% activities respectively after treatment with 2M guanidine-hydrochloride. The hemagglutinating activities of the lectins were abolished completely after treatment with further higher concentration (6M) of guanidine-hydrochloride.

Table-5.5: Effect of Guanidine-HCl at different concentrations on hemagglutinating activities of Sajna leaves lectins.

Concentration of Guanidine- HCl (mole)	Relative hemagglutinating activities (%)		
	SLL-1	SLL-2	SLL-3
0.0	100	100	100
0.25	85	90	85
0.50	60	70	55
1.0	25	35	25
2.0	10	25	15
4.0	5	10	10
6.0	00	00	00

vi) Effect of various metalic salts on the hemagglutinating activities of the lectins.

Table-5.6 represents the effect of various metal ions and salts on the hemagglutinating activities of SLL-1, SLL-2 and SLL-3. From the table it is evident that the activities of lectins abolished completely after treatment with 100mM EDTA solution, while the activities of the lecrins were enhanced significantly in the presence of Ca^{2+} only.

Other metalic salts examined in the study, produced no effect on the hemagglutinating activities of the lectins.

Table-5.6: Effect of various metalic salts on the hemagglutinating activities of Sajna leaves lectins.

Salt added	Concentration (mM)	Relative hemagglutinating activities (%)		
		SLL-1	SLL-2	SLL-3
Control	-	100	100	100
EDTA	100	-	-	-
	50	5	-	-
	20	25	5	-
$CaCl_2$	100	120	135	115
	50	110	110	110
$MnCl_2$	100	100	100	100
	50	100	100	90
Na_2SO_4	100	100	100	100
	50	100	100	100
KCl	100	100	100	100
	50	100	100	100
$MgCl_2$	100	100	100	100
	50	100	100	100

5.8. DISCUSSION

The present study has been carried out to determine the stability of SLL-1, SLL-2 and SLL-3 by using physical and chemical means. The present data concluded that the hemagglutinating activities of Sajna leaves lectins were affected with the changes of pH as well as temperature. Results showed that all the three lectins were more stable in slightly basic pH (i.e. pH 7.2-7.6) than the acidic pH region. The lectins showed maximum activities around temperature 20-35°C and thereafter the activities decreased remarkably with further rise of temperature while the lectins lost their activities completely at 75°C, which might be due to denaturation or disorganization of the structure of lectins at higher temperature.

The biological activities of SLL-1, SLL-2 and SLL-3 lectins were also followed after treatment with acetic acid. Results showed good correlation with those obtained by previous experinents i. e. changes of pH. All the three lectins were found to be inactive almost completely after treatment the native structure of the lectins or ionization of the group located at or near the binding sites. The hemagglutinating activities of Sajna leaves lectins were affected sequentially with the increase in concentration of denaturant such as urea and guanidine-HCl. It was found that the lectins SLL-1, SLL-2 and SLL-3 were more sensitive to guanidine-HCl than urea. All three lectins were inactivated almost completely after treatment with 6M guanidine-HCl whereas in presence of 6M urea, SLL-1, SLL-2 and SLL-3 showed 40%, 45% and 35% activities respectively.

It was also found that the hemagglutinating activities of Sajna leaves lectins were enhanced significantly in the presence of metallic salts Ca^{2+} while in the presence of EDTA, a metal chelator, the activities of lectins were abolished compleltely, suggesting that Ca^{2+} is essential for hemagglutination of Sajna leaves lectins which may be released from the lectins after treatment with EDTA. The inhibitory effect of EDTA on the hemagglutinating activities of TM (Tora- mame) lectin

(Itoh *et. al.*,1980)and mulberry seed lectins have also been reported (Tanzima Yeasmin *et. al.*, 2001).

Some lectins have been reported to be metalloprotein (Goldstein and Hayes, 1978) and a part of metal is essential for the activities of lectins (Takahashi, *et. al.*, 1971; Alford, 1970; Paulova, *et. al.*, 1971a and 1971b and Tunis, 1965).

In Sajna leaves lectins calcium may be present in low concentration and a part of the metal might have been removed from the lectin molecules during the purification steps. This possibility may be supported from the observation that the hemagglutinating activity was increased significantly by the addition of calcium to purified lectins.

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ACKNOWLEDGEMENT

This research was conduct under the direct supervision of well-renewed Professor NURUL ABSAR Department of Biochemistry and Molecular Biology, Rajshahi University, Bangladesh. The author would like to express her deepest respect and appreciation to him for invaluable advice, constructive ideas, immense encouragement and guidance all through the study.

Acknowledgement is also due to Professor Habibur Rahman, Chairman, Department of Biochemistry and Molecular Biology. And thanks to my entire colleague in the aforesaid Department for encouragement and also thanks for the students of Protein and Enzyme Research Laboratory for the technical help. Special deptness is due to Dr. Abbas Ali, Assistant Professor, RUET for his cooperation. It is also great pleasure for the author to express thanks and acknowledgement to Rajshahi University for providing the fellowship.

Grateful to my parents and special gratitude is due to my husband for their inspiration, support and sacrifice throughout the study.

Finally all praise to the 'Almighty Allah' for enabling me to complete this thesis.

The Auther