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Purification, Characterization and Structure-Function Analysis of Proteins from Pota,Nogeton Nodosus Root Stocks

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PURIFICATION, CHARACTERIZATION AND STRUCTURE-FUNCTION ANALYSIS OF PROTEINS FROM *Potamogeton nodosus* ROOT STOCKS

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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CERTIFICATE

This is to certify that the materials included in this thesis are the original research works conducted by **FARZANA PERVIN.** 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 **"PURIFICATION, CHARACTERIZATION AND STRUCTURE-FUNCTION ANALYSIS OF PROTEINS FROM *Potamogeton nodosus* ROOT STOCKS"** 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.

F Pervin 28.8.05

(Farzana Pervin)

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DEDICATED

TO MY

BELOVED CHILDREN

CONTENTS

ABSTRACT

Potamogeton nodosus rootstock contained ash, moisture considerably but significantly higher amount of protein. Rootstock contained smaller amount of lipid as compared to and seeds fruits. It contains carbohydrate such as reducing sugar, non reducing sugar and starch.

Some vitamins are present in the rootstock which include vitamin C, vitamin B_1 and vitamin B_2. Pectin as calcium pectate and crude fibre such as cellulose lignin and pentosan are also present in the rootstock. Some minerals such as calcium, phosphorus and iron are available in the *Potamogeton nodosus* root stock.

The root stock oil was also characterized with respect to its physicochemical properties such as specific gravity, refractive index, smoke point, flash point, fire point, cloud point, solidification point, pour point, saponification value, sapanification equivalent acid value, % FFA, and iodine value.

Six lectins were purified from the root stock of *Potamogeton nodosus*, locally name as Ghechu. The method was accomplished by gel filtration of 100% ammonium sulphate saturated fraction from the crude extract on Sephadex G-50 followed by ion-exchange chromatography on DEAE-cellulose and on CM-cellulose. The purity of lectins were judged by polyacrylamide slab gel electrophoresis. The molecular weight of PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 as determined by Sephadex G-150 and polyacrylamide slab gel electrophoresis method was estimated to be about 98,000, 91,000, 77500, 68,500; 62,500 and 38,500 and 97,500, 83,000; 77,000; 67,500; 62,000 and 38,100 respectively.

PNL-1 is a tetramer in nature and its subunits are held together by non-ionic hydrophobic interactions, PNL-2 was transformed into single band corresponding to a molecular weight of 36,200 indicating that the protein

contains four subunits of equal sizes, which are held together by S-S bond. PNL-3 and PNL-4 are dimer in nature which are held together by disulfide bond (s), while PNL-5 and PNL-6 contain only one subunit i.e. they are monomer in nature. The lectins agglutinated specifically by rat red blood cells and the agglutination was inhibited specifically D-mannose, methyl-α-D-mannopyranoside and methyl-β-D- mannopyranoside. The lectins PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 contain 3.22, 4.0, 3.75, 3.72, 3.4 and 3.10% neutral sugar respectively and the sugar composition of the lectins were D-galactose for PNL-1 and PNL-6, mannose for PNL-2, PNL-3 and PNL-4 and D-glucose for PNL-5.

The lectins exhibited cytotoxic effect in brine shrinp lethality bioassay. The biological activities of the lectins PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 were investigated 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 6.5-7.2 and temperature 20-35°C.

The biological activities of the lectins were abolished with the higher concentration of acetic acid and denaturants, guanidine-HCl and urea. The activities of all the lectins were affected more profoundly by guanidine-HCl than urea.

The biological activities of the lectins were enhanced significantly in the presence of Ca^{2+} and Cu^{2+} while the activities were abolished completely in the presence of EDTA.

LIST OF ABBREVIATIONS

DEAE-Cellulose	Diethylaminoethyl cellulose
BSA	Bovine serum albumin
CM- Cellulose	Carboxymethyl cellulose
PBS	Phosphate buffer saline
ASTM	American Society for Testing and materials
PNL-1	*Potamogeton nodosus* lectin-1
PNL-2	*Potamogeton nodosus* lectin-2
PNL-3	*Potamogeton nodosus* lectin-3
PNL-4	*Potamogeton nodosus* lectin-4
PNL-5	*Potamogeton nodosus* lectin-5
PNL-6	*Potamogeton nodosus* lectin-6
TLC	Thin Layer Chromatography

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CHAPTER ONE

Introduction

1.1. 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. Starch and sugars, furnish most of the energy we need to move, perform work and keep alive. Fats and oils are concentrated form of energy and are important as carrier of certain vitamins. Their essential fatty acids are needed for normal growth and healthy skin. Proteins in the diet provide essential amino acids and nitrogen required for normal growth and maintenance of body tissues. In addition, antibodies, enzymes and certain hormones are produced in the body from amino acids available in proteins of the diet. Vitamins, although needed only in minute quantities in the diet, are essential as co-enzyme of many enzymes catalyzing and regulating vital biochemical reactions within cells. Besides these, plants also offer us such substances as glycosides, alkaloids, sterols, saponins, essential oils, resins, tannins etc. Some of which, in regulated doses are effective remedies for many diseases. Plants also help us to maintain air environment. Plants containing medicinal ingredients are usually classified as medicinal plant (Jesmin, M. *et al.*, 1991).

From the dawn of human civilization, plants have been used for the treatment of various diseases. It has been pointed out that even today about 75% of the world population still relies on plants, plant extracts and other tool of traditional medicines (Abelsons, 1990). Yet a possible total of 250,000 to 300,000 plants species, most of them tropically inhabited, only some 5,000 have so far been tested exhaustively for their medical applications.

The science of medicine is very old. Plants and plant products have been used to alleviate the sufferings of the body and mind from time-immemorial (Core, E. L. 1962). The root of drug uses lie in antiquity. Some ancient Chinese Emperors and Roman Philosophers used empirical methods of collection and therapeutic administration of herbs and herbal concoctions that evoke an echo in the present-day selection and medical study of botanical drug mixtures. The oldest records of therapeutic plants from the ancient civilizations of the Chinese, the Hindus, the South American Mayas, and the Mediterranean peoples of antiquity. The scholar-Emperor Shen-Nung (2735 B.C.) compiled a book of herbs and is said to have observed the antifebrile effects of *chlang shang*, which has been shown to contain antimalarial alkaloids in modern times. He also noticed the diaphoretic and stimulatory effect of the drug *ma huang,* from which, almost 5000 years later, Nagai isolated the active alkaloid ephedrine. Other oriental civilizations have also left us records of their uses of medicinal plants. The antileprotic action of the *chaulmoogra* fruit was known to the ancient Indians, and the *ipecacuanha* root was used in Brazil and the Far East for treatment of the dysentery and diarrhoea. Its main alkaloid, emetine, still constitutes an important drug for amebiasis. The South American natives knew the paralytic effects of curare (Alfred Burger, 1960) and used it as arrow poison. Tubocurarine (British Pharmaceutical Codex; 1963), a quaternary alkaloid from curare, is now used as an adjunct to anesthesia in surgery and in obstetrics.

One great herbal drug of the seventeenth century has survived the test of time, the cinchona bark from which quinine was later extracted. It was brought to Europe from South America by missionaries (hence the name Jesuit bark or powder), and after being rejected initially by unconvinced medical faculties, it became the prize medicine for fevers, chills and malaria's.

Although the importance of investigation was realized by workers and the therapeutic properties of various plant products have been known and utilized from early time, it was not until 1803, that the first physiologically active principle isolated, was a crude of the alkaloid, narcotine, which Derosne (Ferguson) obtained from a syrupy extract of opium. This beginning opened up an era of successful isolation of a large number of other physiologically active principles of plant origin. Morphine was isolated from opium by Sertuner (Alfred Burger, 1960) in 1806, quinine by Pelletize and Caventou (R.H.F. Manske, *et al.*) in 1920, and the mydriatic base atropine by Mein Geiger and Hesse (Alfred Burger, 1960) in 1933. In 1864, physostignine was isolated from calabar bean by Jabst and hesse (Henry). This was followed by the isolation of the anthelmintic alkaloid pelletierine (Alfred Burger, 1960) from pomergranate in 1877 and of ephedrine (M.F. Hesse *et al.*, 1966) from *Ephedra vulgaris* in 1887. Many other chemical compounds have since then been isolated and their chemistry and pharmacological action studied. The discovery of the medicinal plants of Indian subcontinent was made hundreds of years ago (Chatterji Ashima, 1976). Rigveda, Atharvaveda, Ayurveda, Susruta and Charaka, are amongst the oldest treaties of Indian medicinal plants. In Indian literatures, many ancient plants have been described, of which the use of croton as a purgative; the smoking of Datura in case of asthma and the use of Nuxvomica in paralysis and dyspepsia are important. The antileprotic effect of *chaulmoogra* oil was also known to the ancient Indians.

During the middle ages when the Romanian glory began to decline, the arabs contributed much to the vast field of medicine. Canons of medicine by Ibnsina bears testimony to this. With the advent of the Muslim conquerors, the Mohammadan system of medicine was introduced in the subcontinent and the system of medicine was of superior standard and was fairly advanced for the period. An excellent account of the medicinal plants of the Indian subcontinent can be seen in the book written by Abu Mansur in that period (Core, E. L. 1964).

Our country abounds with a host of medicinal plants and herb's. Nature has endowed Bangladesh with enormous plant resources. Many of these plants grow wild in jungles, forests, garden, pools, marshy lands and many of them are found lying every where in the fertile region of the country. Some of these plants are used in Ayurvadie and Hekimi system of medicine prevalent in the country. Of the large number plants used in indigenous system of medicine, there are many that deserve the reputation, they have earned as cures while others may have little or no effects. Studies of some of these plants in the recent past have been rewarding. In 1952, Schlittler and coworkers isolated Reserpine from *Rauwolfia serpentina* Benth, which grows wild in Bangladesh. The discovery of Reserpine as effective high potensive and tranquilizing agent has reemphasized research in plants for medicinal agents

Researches in medicinal plant products have been continuing. Current researches in this field do not, however, limit themselves any more to more isolation and characterization of the active principles. Much attention is now paid to the study of their mode of action, structure-activity relationship and biosynthesis. The information available from such studies may help as design better therapeutic agents, hopefully more potent, more specific, and less toxic.

Although the works on medicinal plants cover only but a small fraction of the vast medicinal plants population of the country, these show definitely that researches in medicinal plants in this country is receiving more attention than before. This is indeed a very happy sign for the future.

1.2 DISTRIBUTION:

Bangladesh being situated in the monsoon area of the world, has vast treasure of indigenous plants of various families, most of which grow wild in forests, jungles, hillocks and gardens. Different segments of these plants such as roots, stems,

leaves, barks, flowers, seeds etc. have been used by the Hekims, Kabirajes and Vaidayas as successful remedy for various diseases since the ancient time. The importance of sarpagandha (*Rauwolfia serpentina*) as source of anticancer drugs, vinblastine and vincristine has re-stimulated research in medicinal plants for remedying many hitherto incurable diseases and researches in indigenous medicinal plants are in progress in different laboratories of the world.

The works mentioned above cover only a small fraction of the vast medicinal plant population of the country and do not include *Potamogeton nodosus*, a member belonging to the genus Potamogeton of the family Potamogetonaceae and growing mainly in the districts, Tangail, Chuadanga, Cox's Bazar, Dhaka, Munshiganj, Mymensingh, Rangpur, Naogaon and Rajshahi of Bangladesh. Also distributed throughout the plains of India, ascending the Himalayas to 9000 ft. in Sikkim, Burma, Malaya, Sandwip Island, Srilanka and Malaysian region.

1.3 BOTANY:

a) The plant family -- Potamogetonaceae [Mc Lean, R.C. & Ivimey Cook, W.R. 1961; Khan M. Salar & Mahbuba Halim, 1987]:

The plant under investigation is *Potamogeton nodosus* Poir which belongs to the family Potamogetonaceae. The potamogetonaceae are a small family of about ten genera which are generally submerged water plants, a few having floating leaves. Several genera are found in sea water. Rootstocks creeping, usually rooting at the lower nodes. The stem usually consists of a rhizome anchord by adventitious roots arising at the node. The leaves (example *Potamogeton natans*) are produced alternately and consist generally of a board, entire lamana borne on a long petiole, or they may consist merely of a linear blade. Intravaginal scales occur in the leaf axils. The flowers are either monoecious or diecious; the floral parts are either dimerous or tetramerous but a perianth is generally absent. In many species the flowers are born above water. The anthers are sessile and the carpels free with

solitary ortotropous or campylotropous ovules. In potamogeton the four anthers bear petaloid outgrowths from the back of the connectives, stimulating a perianth. The inflorescence of zostera consists of a linear series of alternating carpels and stamens, the whole being enclosed by the sheathing margins of a leaf.

Special organs of vegetative propagation occur in some species and consist of tuber bearing runners. Anatomically the plants have the typical structure of aquaties with a well-developed aerenchyma. It has been found that the quantity of sclerenchyma produced varies with the habitat, for plants growing in running water have more mechanical tissue than those found in stagnant water. In marine species the mechanical tissue is well developed and air spaces may be absent.

Pollination in Potamogeton (example *Potamogeton crispus*) is anemophilous and the grains are round, but in other genera, such as zoster (example *Zostera nana*), the pollen is filamentous and develops into alga - like threads of the same specific gravity as the water. These filaments become entangled in the ranching stigmas (example - *Zostera marina*) and pollination effected. This type of hydrophilous pollination is rare and has been only imperfectly studied.

The family includes a number of genera of which potamogeton is the largest. Among the other genera, Ruppia and Zannichellia, (example - *Zannichellia polycarpa*) which are monotypic, and two of the six species of zostera are found in British waters. Other genera occur all over temperate and subtropical regions of both hemispheres.

b) The Plant Genus - Potamogeton [Khan M. Salar & Mahbuba Halim, 1987; Hooker, J. D. 1894]:

A large genus of potamogeton is distributed in the temperate and tropical regions of the world. The species are difficult to separate but over twenty have been recognized in Britain. About fifteen species are recorded in India. The plants of

the genus slender herbs with creeping rootstocks, found nearly throughout Indian subcontinent, inhabiting freshwater tanks, pools, marshy lands, old ponds and canals, often growing gregariously. They bear submerged or floating leaves and minute flowers in auxiliary spikes. The fruits are small and indehiscent.

The plants are of little economic value; they serve at the most as food for some wild water fowls, ducks and as shelter and shade for fishes. Often they support growth of algae and small animals, which indirectly provide food for game fishes. Sometimes they are said to help in softening water by removing lime and carbon dioxide (CO_2) (Fasset, 348-49, 357-58).

c) Members of Potamogetonaceae available in Bangladesh [Khan, M.S. and Rahman, M. Matiur, 1989].

Among the members of potamogetonaceae, only a few are denizens of Bangladesh. The following members are available in Bangladesh.

1. *Potamogeton crispus* Linn.
2. Potamogeton *mucronatus,* Presl Epinel.
3. *Potamogeton nodosus* Poir, (Synonym:*Potamogeton indicus* Roxb.)
4. *Potamogeton octandrus* Poit,
5. *Potamogeton pectinatus* Linn.

d) The plant species - *Potamogeton nodosus* Poir [Hooker, J. D. 1894; Haq. A. M. 1986; David prain 1963; Haq, A.M. 1986; Biswas, Shri Kalipado & Ghosh, Shri Ekhari 1977]:

Potamogeton nodosus Poir is one of the plant among 15 species found in India. Its synonym is *Potamogeton indicus* Roxb. *Potamogeton nodosus* Poir is a very common aquatic herb. It is found in all the provinces of Indian subcontinent as well as in Bangladesh.

It is a submerged water plant with creeping rootstock. Generally it grows pools, marshy lands, old ponds, canals and inhabiting fresh water tanks. Its upper floating leaves are large, elliptic or elliptic - lanceotate corriaceous, lower submerged leaves are longer narrower membranous, stipules 1-1/2 in free, spike dense-fid, druplets small smooth. It contains rootstock. Its fruits are small and it looks like long pepper. Its new leaves grow in August - October. This plant acts as a nutrient for many fishes. It is a favorite food of shoal fish.

e) Taxonomy of Potamogetonaceae [Khan M. Salar & Mahbuba Halim, 1987; Hooker, J.D. 1894]:

They are submerged water plants with creeping rootstock. Leaves submerged or floating, opposite or alternate, entire or toothed; stipule intrafoliaceous. Flowers small spicate on a space rising from a membeanous spathe, ebracteate, perianth-segments 4, concave, green, valvate. Sten branched. Inflorescence of axillary spikes, emerged or submerged. Anthers 4, sessile on the segments, didymous, slits extrose. Tepals 4, stamens 4, carpels 4; ovary superior, style short; stigmas sessile, 1-celled, lovuled, stigma, subsessile or decurrent, persistent; ovule inserted in the inner angle of the cell; campylotropous. Fruit of 4, somewhat compressed drupelets. Drupelets small, coriaceous or spongy. Seed subreinform; embryo macropodal. Seeds with well developed hypocytyle and no endosperm.

f) Botanical features of *Potamogeton nodosus* [Khan, M. Salar & Mahbba Halim, 1987; Hooker, J.D. 1894; Haq, A.M. 1986; Biswas, Shri Kalipodo & Ghosh, Shri Ekhari 1977; Dr. Hasan, Md. Aul and Huq Ahmed Mozharul 1993; Dr. Rahman, S. Hossain, A.K.M.Z., Rahman, M.A., Dr. Mutaleb, S.A. 1997].

Family	:	Potamogetonaceae
Genus	:	Potamogeton
Scientific name	:	*Potamogeton nodosus* Poir.
Synonym	:	*Potamogeton indicus* Roxb.

Vernacular name	:	1) Bengali name: Paney pippol 2) Sanskrit name: Jal pippoly. 3) Local name: Ghechu.
Diagnostic features	:	It possesses almost all characteristics of Potamogetonaceae.
Habit	:	Submerged aquatic herb
Leaves	:	Upper leaves (3-4.5 by 1-2.5 inch) floating oblong elliptic or elliptic - lanceolate coriaceous, lower submerged longer narrower membranous, stipules 1-1.5 inch. Free, spike dense-fid, drupelets small smoth.
Rootstock	:	It is underground metamorphosed stem. It grows vertically rather than horizontally in marshy lands.
Fruits	:	Fruits are small and it looks longpepper.
Used portion	:	Leaves, fruits and rootstock.
Place of birth	:	Pools, marshy lands, old ponds, canals and inhabiting freshwater tanks.

1.4. PLANT ROOTSTOCKS AS A RICH SOURCE OF PROTEIN:

Although animal kingdom is the main source of protein but plant kingdom also possesses a lot of protein. Green leaves, barks, roots, stems etc. contain small amount of protein; while rootstocks and seeds, are the main sources of protein in plants. Pulses contain about 13-16% and oil seeds contain approximately 30-55% protein (Rutkowaski, 1970). In addition, wheat, barley, rice bran, maize, etc. also contained significant amount of protein. Some plant rootstock and seed proteins are toxic and some are non toxic.

1.5. MOST OF THE PLANT PROTEINS ARE GLYCOPROTEIN:

The glycoprotein which contain carbohydrate group attached covalently to the polypeptide chain represent a large group of wide distribution with considerable biological significance. Plant seed proteins contain in general 1-3% carbohydrate, plant rootstock proteins contain in general 1-5% carbohydrate but there are exceptional cases in which the carbohydrate content may be about 10-12% (Lis and Sharon, 1981). The percent by weight of carbohydrate group in different glycoprotein may vary from less than 1% in ovalbumin to as 80% in the mucoprotein (Lehninger, 1981).

Many different types of monosaccharide derivatives have been found in glycoprotein. The linear or branched side chain of glycoprotein may contain from two to dozen of monosaccharide residues usually of two or more kinds. Some glycoproteins also contain oligosaccharides units. The sugar residues are generally mannose, galactose, lactose, xylose, glucose, raffinose, glucosamine etc. (Lis and Sharon, 1981). Among them mannose and glucosamine are predominant.

1.6. LECTIN:

Lectins, a class of proteins that bind sugar specifically and reversibly which agglutinate red blood cells. The term lectin was proposed by Boyd, W.C. (1970) because of their unique carbonhdyrate binding properties.

Lectins are widely distributed in the plant kingdoms, particularly among the legumes and to a lesser extent among the cereal grains. More than 90 lectins from leguminous plants belonging to differen suborders and tribes have been isolated, mostly from seeds, and characterized to varying degree. Lectins are also found in animals. Insects, and microorganisms (Sharon and Goldstein, 1986; Lis and Sharon, 1986; Sharon and Lis, 1989). Moreover, it has become clear that lectins

are present in some invertebrate animals such as snails (Sharon, 1987). Recently Vivian Teichberg (1975) at the Weizmann Institute of Science of Israel discovered a lectin in the electric eel. These substances have also been referred to as Phytohemagglutinins, although the term phytolectins has been proposed in order to distinguish those lectins which are found in plants from those which are of animal or microbial origin.

1.7. CLASSIFICATION AND PROPERTIES OF LECTINS:

Lectins are usually classified on the basis of source. They are of various types : plant lectin, vertebrate lectin, invertebrate lectin, lectin of slime mold, lectin of protozoa, viral lectin and bacterial lectin according to their respective source.

Regardless of source all of the effects of lectins are believed to a manifestation of the ability of the lectins to specific kinds of sugar, thereby agglutinate red blood cells of various animals and possess many other characteristic properties such as exert wide range of biological effects on the cell. Some of the properties of each group of lectin are given below:

1.7.1. PLANT LECTIN:

Plant lectins are the first and still the largest and best characterized group. It becomes apart that many of the lectin could be grouped into families with sequence homologies and common structural properties. The largest and best characterized family is that of the Leguminoseae lectins. Two small families, also of plant lectins, are those from *Gramineae* (cereals) and *Solanaceae* (e.g. potato and tomato) lectin (Sharon and Halima, 1990). The main source of lectins is mature seeds. Similar to the major reserve protein of the seeds, the bulk of the lectin is located in the cytoledons in organelles known as protein bodies (Etzler, 1986; Chrispeels and Tague, 1990). Small amount of lectin are present in other tissues such as leaves, bark, and roots (Etzler, 1986; Chrispeels *et al.*, 1900;

Person *et al.*, 1987; Schenell and Etzler, 1988). 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, L-fructose, N-acetyl glucosamine, N-acetyl galactosamine, galactose, and N-acetyl neuramic acid) according to the monosacharide that is the most effective inhibitor of the agglutination of erythrocytes or precipitation of carbohydrate containing polymers by the lectin.

1.7.2. VERTEBRATE LECTIN:

Vertebrate lectins are divided into two classes, these are:

(a) Integral membrane lectins that require detergents for their extraction; and

(b) Soluble lectins (Barondes, 1981; Barondes, 1984).

The first group comprises of lectins that differ in their sugar specificities (mannose, L-fructose, mannose-6-phosphate and N-acetyl galactosamine) and physicochemical properties (Ashwell and Harford, 1982; Mosigny *et al.*, 1983). Among the best characterized lectins of this class are the galactose and mannose / N-acetyl glucosamine specific receptors of mammalian and avian hepatocytes, respectively (Drickamer, 1985), and the receptor for the mannose-6-phosphate present in a variety of cells (Hasilik and Vonfigura, 1986).

Of the soluble vertebrate lectins, the first to be purified was the ß-galactoside specific lectin from the electric organ of the eel *Electricus electricus* (Teichberg *et al.*, 1975; Levi and Teichberg, 1981). The soluble ß-galactoside specific vertebrate lectins are of a similar molecular size, consisting each of two subunits of M.W. 13,500 to 16,500 (Baondes, 1984) and require a reducing agent to maintain their carbohydrate binding activity.

1.7.3. INVERTEBRATE LECTINS:

These lectins are found in practically all of the approximately 30 phyla and the various classes and subclasses of invertebrates (Cohen, e, ed. 1984; Gold and Balding, 1975), mainly in the hemolymph and sexual organs, e.g. albumin glands and eggs (Yeaton, 1981; Gilboa-Garber *et al.*, 1985). They also present in the membrabes of hemocytes, cells that function as primitive and rather unspecific immunological protectors (Cheng *et al.*, 1984; Vasta *et al.*, 1984).

1.7.4. THE BACTERIAL SURFACE LECTINS:

Many intact bacteria possess the ability to bind and agglutinate erythrocytes and other type of cells (Mirelman, 1986; Sharon, 1986). These activities are frequently inhibited by sugars, suggesting the presence of lectins on the bacterial surfaces (Sharon *et al.*, 1977).

1.8. BIOLOGICAL ROLES AND APPLICATIONS OF LECTINS:

Lectins are currently attracting much interest, primarily because they serve as invaluable tools in diverse areas of Biomedical research. Because of their unique carbohydrates binding properties, lectins are useful for the separation and characterization of glycoproteins and glycopeptides, in studies of glycolipids; following changes that occur on cell surfaces during physiological and pathological processes, from cell differentiation to cancer; histochemical of cells and tissues; tracing neuronal pathways; typing blood cell and bacteria; and for fractionation of lymphocytes and of bone marrow cells for bone marrow transplantation.

Lectins are also used to stimulate lymphocytes to asses the immunostate of patients and for chromosome analysis in human cytogenetics, as well as for the production of cytokinase.

In addition, lectins are excellent models for examine the molecular basis of specific reaction that occur between proteins and other types of molecules, both of low or high molecular weight, such as the binding of antigens to antibodies, of substrates to enzymes, of drugs to proteins and of hormones and growth factors to cells.

Table 1.1: Biological roles of lectin in nature

Source	**Roles**
Plants	(a) Attachment of nitrogen-fixing bacteria to legumes.
	(b) Protection against phytopathogens.
Animals	(a) Endycytosis and intra-cellular translocation of glycoproteins.
	(b) Regulation of cell migration and adhesion
	(c) Recognition determinants in nonimmune phagocytosis.
	(d) Binding of bacteria to epithelial cells
Microorganisms	(a) Attachment of bacteria and parasites (e.g. amoeba and plasmodium) to host cells.
	(b) Recognition determinants in nonimmune phagocytosis.
	(c) Recognitition determinants in cell adhesion of slime molds.

1.9. THE TOXIC EFFECT OF LECTINS AND ITS ROLE AS ANTICANCER AGENTS:

Both oncogenic (Abu *et al.*, 1963; Burger, 1969; Inbar and Sachs, 1969; Acla *et al.*, 1970) and non oncogenic (Zarling ad Tavethia, 1971) viral transformed cell possess increased susceptibility to agglutination by lectin, that exhibit as a toxic substance even in very small amount to higher animals. Abrin and Ricin possess such kinds of characteristics. They inhibit protein synthesis (Lin, *et al.*, 1970) as well as DNA and also RNA in the cell of culture.

A tumor inhibiting effect of Ricin was reported (Mosinger, 1951). Lin *et al.*, (1969) demostracted that Abrin and Ricin were able to prevent the development of ascites tumor even when administered five days after inoculation of the tumor cells. The toxins have also been tried in the treatment of human cancer, particularly uterine cancer (Tung *et al.*, 1971).

1.10. MEDICINAL IMPORTANCE OF *Potamogeton nodosus* Poir [Shri Ekhari 1977]:

Potamogeton nodosus Poir is locally credited with medicinal properties and has found uses in folk medicine as a cure for various diseases. Still this plant is widely used as Ayurvedic medicine in Indian subcontinent. Juice of this plant is bitter which is used for treatment of diarrhoea and dysentry. Juice of its leaves are used in cough and antibilious. Its fruits are used in the treatment of cough and antiflatulants. The plant is used as antipimplious. The powdered rootstock is given in dyspepsia, diarrhoea, dysentry and piles. The powdered rootstock are also used widely in rural areas of Bangladesh in the treatment of Jaundice.

In Ayurvede *Potamogeton nodosus* an aquatic plant, was reported as an effective agent for the treatment of a variety of disease of different etiology, such as,

1) Cough,
2) Tuberculosis,
3) Cancer (Bisharpo or horkat as they called),
4) Acne,
5) Diarrhoea,
6) Dysentry,
7) Piles,
8) Dyspepsia,
9) Antibilious,
10) Antiflatulants,
11) Jaundice.

1.11. LITERATURE REVIEW:

Shahanaz Khatun (2005) extracted and purified three lectins from Sajna (*Moringa oleifera* L.) leaves by gel filtration of 100% ammonium sulfate satmated crude protein extract followed by ion exchange chromatography on DEAE cellulose and affinity chromatography on Sepharose-4B. The MW of the lectins as determined by gel filtration were 1,55,000; 1,15,000 and 85,000 for SLL-1, SLL-2 and SLL-3 respectively and the MW as determined by SDS-polyacrylamide slab gel electrophoresis were 160,000, 1,20,000 and 85,500 respectively. SLL-1 gave a strong band corresponding to molecular mase of 1,00,000 and a slightly faint band corresponding a molecular mase of 60,000 and SLL-2 gave two bands corresponding to a molecular mass of 65,000 and 55,000 while SLL-3 gave two bands corresponding to a molecular weight of 85,000. The lectins agglutinated rat blood cells and this agglutination was inhibited by galactose and galactose containing saccharides. The lectins exhibited strong cytotoxic effect in brine shrimp lethality bioassay.

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.

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 hemag-glutinating 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.

1.12. AIM OF PRESENT INVESTIGATION:

Lectins are widely distributed in nature. Till now, most of the lectins are purified from plant sources and the main sources of lectins in plants are mature seeds. Researches on lectins are currently attracting much interest to the scientist because of their unique biological properties. Although hundred of lectins are known but the structure of most lectins have not yet been fully elucidated. However, the physiological function(s) of plant lectins is still unknown.

Rootstocks of *Potamogeton nodosus* locally name as Ghechu has been used as food by the poor people of our country in the rural areas. Sometimes news appeared in the daily news papers about death of people by eating Ghechu as a sole source of food. Ghechu may be considered to use as a supplementary sources of protein, if possible, since it contains about 13-16% protein.

It was already known that plant materials contain mostly glycoprotein which are toxic in nature. Keeping this view in mind, in the study, we have tried first time to purify the proteins specially lectin from Ghechu and also tried to do researches to obtain more information about nature of the protein.

The present thesis includes the following research works,

(I) Determination of the chemical compositions of the rootstocks of *Potamogeton nodosus*.

(II) Purification and characterization of proteins specially lectins from rootstocks of *Potamogeton nodosus*.

(III) Effect of physical and chemical agents on the stability of the lectins purified from *Potamogeton nodosus*.

Fig. 1.1: Photograph of the Rootstock of *Potamogeton nodosus* Poir.

Fig. 1.2: Photograph of the Ghechu of (*Potamogeton nodosus* Poir.) Plant.

CHAPTER TWO

An analysis of Physico-Chemical Compositions of *Potamogeton nodosus* Root Stocks

2.1. INTRODUCTION:

Potamogeton nodosus Poir is one of the most popular and widely available aquatic herbs in Bangladesh. Its rootstocks supplies ready sources of carbohydrate, protein, lipid as well as other nutrients. The poor people of our country in the rural area use the rootstocks as additional food. The plants are of little economic value; they serve as food for some wild water-fowls, ducks and as shelter and shade for fishes. Often they support growth of algae and small animals, which indirectly provide food for game fishes. The primary aim is to determine the biochemical constituents of the rootstock of *Potamogeton nodosus* Poir which locally called as Ghechu or Pondweed.

2.2. MATERIALS AND METHODS

2.2.1. DETERMINATION OF MOISTURE CONTENT OF THE ROOTSTOCK:

Moisture was determined by the conventional procedure.

Materials:

Porcelain crucible, Electrical balance, Oven and Desiccator.

Procedure:

One to two gms of rootstocks were weighed in a porcelain crucible (which was previously cleaned and heated to 100°C, cooled and weighed). The crucible with the sample was heated in an electrical oven for about six hours at 100° C. It was then cooled in a desiccator and weighed again.

Calculation:

Percent of moisture content (gm per 100 gms of rootstock)

$$= \frac{\text{Amount of moisture obtained}}{\text{Weight of rootstock}} \times 100$$

2.2.2. DETERMINATION OF ASH CONTENT OF ROOTSTOCK:

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

Materials:

Porcelain crucible, Electrical balance, Desiccator and Muffle furnace.

Procedure:

Four to six gms of rootstocks were weighed in a porcelain crucible (which was previously cleaned and heated to about 100^0C, cooled and weighed). The crucible with its content was placed in a muffle furnace for 4 hours at about 600^0C. This was then cooled in a dessicator and weighed. To ensure completion of ashing, the crucible was again heated at the same temperature in the muffle furnace for half an hour, cooled and weighed again. This was repeated till two consecutive weights were the same.

Calculation:

Percent of ash content (gm per 100 gms of rootstock)

$$= \frac{\text{Amount of ash obtained}}{\text{Weight of rootstock}} \times 100$$

2.2.3. DETERMINATION OF LIPID CONTENT OF ROOTSTOCK:

Lipid contents of rootstocks were determined by the method of Bligh and Dyer (1959).

Reagents:

A mixture of chloroform and ethanol (2: l, v/v)

Procedure:

About one gm of rootstock was first grounded in a mortar with about 10 ml of distilled water. The grounded flesh was transferred to a separating funnel and 30 ml of chloroform- ethanol mixture was added. The mixture was mixed well and then kept overnight at room temperature in the dark. At the end of this period 20 ml of chloroform and 20 ml of water were further added and mixed well. Generally three layers were seen. A clear layer of chloroform containing all the lipids, a colored aqueous layer of ethanol with all water soluble materials, and a thick pasty interphase were seen.

The chloroform layer was carefully collected in pre-weighed beaker (50 ml) and then placed on a steam bath for evaporation. After evaporation of the chloroform, the weight of the beaker was determined again. The difference in weight gives the amount of lipid.

Calculation:

Percent of lipid content (gm per 100 gms of rootstock)

$$= \frac{\text{Amount of ash obtained}}{\text{Weight of rootstock}} \times 100$$

2.2.4. DETERMINATION OF TOTAL SUGAR CONTENT OF ROOTSTOCK:

Total sugar content of rootstock was determined colorimetrically by the anthrone method (Jayaraman, 1981)

Reagents:

Anthrone reagent: The reagent was prepared by dissolving 2 gms of anthrone in one litre of concentrated sulphuric acid (H_2SO_4).

Standard glucose solution: A standard solution of glucose was prepared by dissolving 10 mg of glucose in 100 ml of distilled water.

Preparation of sugar extract from rootstock:

Sugar extract from rootstock was prepared by the following method, (Loomis and Shull, 1937).

Four to six gms of rootstocks were cut into small pieces and grinded into paste using mortar and pastle and immediately placed into boiling ethyl alcohol and allowed to boil for 5 to 10 min (5 to 10 ml of alcohol was used for every gm of rootstock). The extract was cooled and crushed thoroughly again in a mortar with a pestle. Then the extract was filtered through two layers of muslin cloth and re-extracted the ground tissue for three minutes in hot 40% alcohol using, 2 to 3 ml of alcohol for every gm of tissue. This second extraction ensured complete removal of alcohol soluble substances. The extract was cooled and passed through muslin cloth. Both the extracts were filtered through Whatmann no. 41 filter paper.

The volume of the extract was evaporated to about $1/4^{th}$ the volume over a steam bath and cooled. This reduced volume of extract was then transferred to a 100 ml volumetric flask and made upto the mark with distilled water. Then 1 ml of the diluted solution was taken into another 100 ml volumetric flask and made upto the mark with distilled water.

Procedure:

Aliquot of 1 ml of extracted solution was pipetted into test tube and 4 ml of the anthrone reagent was added to each of this solution and mixed well. Glass marbles were placed on top of each tube to prevent loss of water by evaporation. The tubes were placed in a boiling water bath for 10 min, then removed and cooled. A

reagent blank was prepared by taking 1 ml of water and 4 ml of anthrone reagent in a test tube and treated similarly. The absorbance of the blue green solution was measured at 620 nm in a spectrophotometer. Glucose solution in different test tube containing 0.0, 20µg, 40 µg, 60µg, 80µg and 100µg of glucose were taken and made the volume upto 1.0 ml with distilled water. Then 4 ml of anthrone reagent was added to each of the test tube and mixed well. All these solution were treated similarly as described above. The absorbance was measured at 620nm using the blank containing 1 ml of water and 4 ml of anthrone reagent.

Calculation:

The amount of total sugar present in the extracts was calculated from the standard curve of glucose (Fig. 2.1). Finally, the percentage of total sugar present in the rootstock was determined using the formula given below:

Percentage of total sugar (gm per 100 gms of rootstock)

$$= \frac{\text{Amount of sugar obtained}}{\text{Weight of rootstock}} \times 100$$

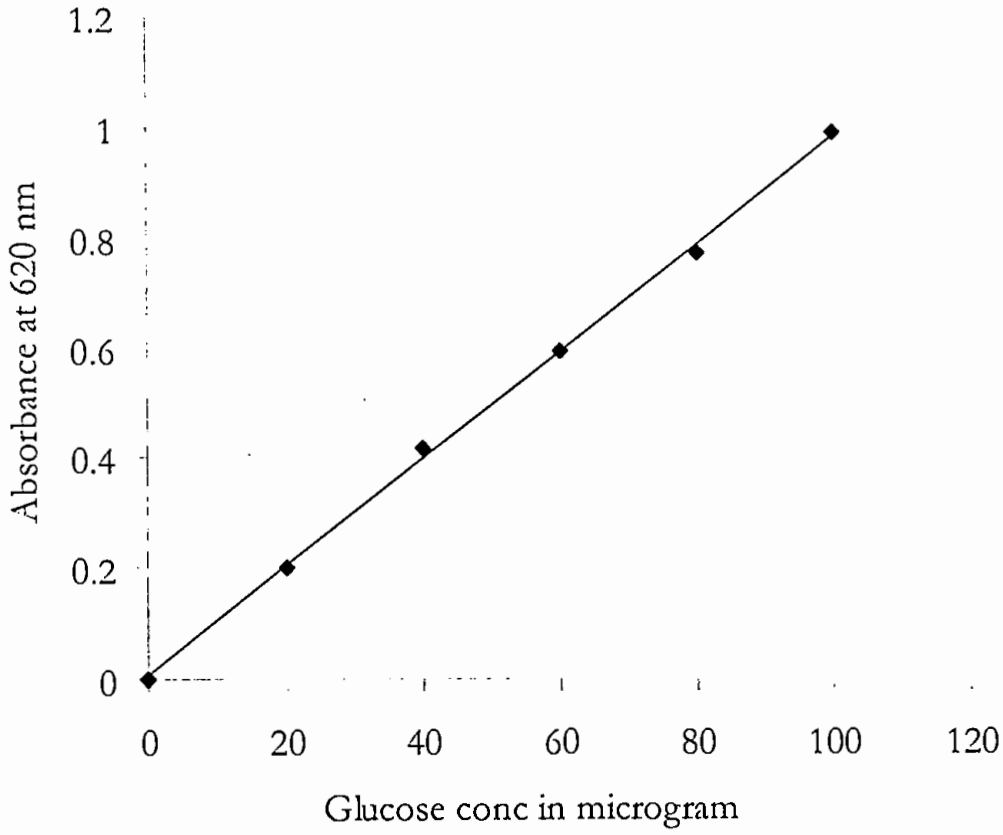


Fig. 2.1. Standard curve of glucose for estimation of total sugar and starch.

2.2.5. DETERMINATION OF REDUCING SUGAR CONTENT OF ROOTSTOCKS:

Reducing sugar content of the rootstock was determined by dinitrosalicylic acid method (Miller, 1972).

Reagents:

Dinitrosalicylic acid (DNS) reagent: Simultaneously 1 gm of DNS, 200 mg of crystalline phenol and 50 mg of sodium sulphite were placed in a beaker and mixed with 100 ml of 1% NaOH solution by stirring. If it is stored then sodium sulphite is added just before use 40% Rochelle salts.

Preparation of sugar extract from rootstock:

Sugar extract from the rootstock was prepared following the procedure as described in section, 2.2.4.

Procedure:

Aliquot of 3ml of the extract was pipetted into test tubes and 3ml of DNS reagent was added to each of the solution and mixed well. The test tube was then heated for 5 min in a boiling water bath. After the color has developed, 1 ml of 40% Rochelle salt was added to each of the tubes, when the contents of the tubes were still warm. The test tubes were then cooled under a running tap water. A reagent blank was prepared by taking 3ml of water and 3ml of DNS reagent in tube and treated similarly. The absorbance of the solution was measured at 575 nm in a colorimeter.

The amount of reducing sugar was calculated from the standard curve of glucose (Fig. 2.2).

Calculation:

The percentage of reducing sugar (gm per 100 gms of rootstock)

$$= \frac{\text{Amount of reducing sugar obtained}}{\text{Weight of rootstock}} \times 100$$

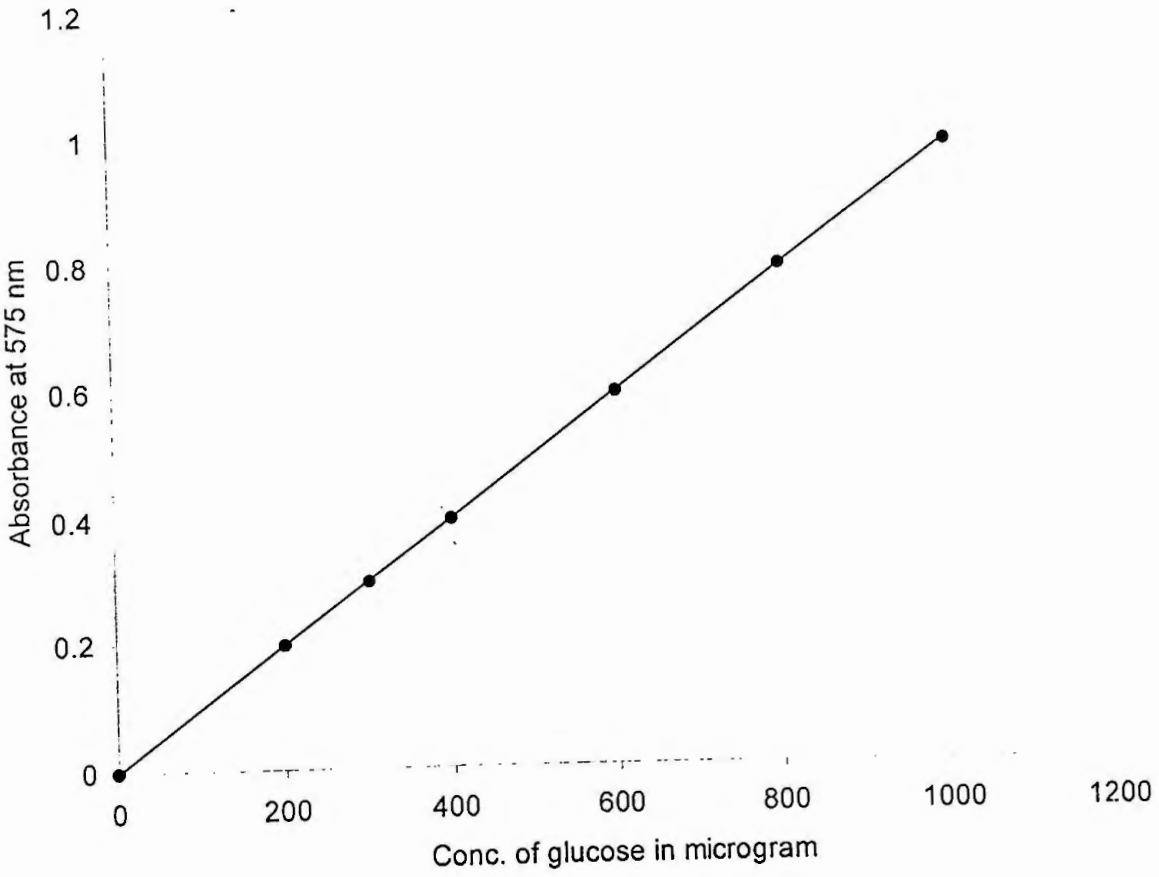


Fig. 2.2. Standard curve of glucose for estimation of reducing sugar.

2.2.6. DETERMINATION OF NON-REDUCING SUGAR CONTENT OF ROOTSTOCK:

Non-reducing sugar (sucrose) content was calculated from the following formula (Ranganna, 1979).

% Sucrose or non reducing sugar = (% Total sugar - % Reducing sugar) × 0.95.

2.2.7. DETERMINATION OF STARCH CONTENT OF ROOTSTOCK:

The starch content of rootstock was determined by the anthrone method (Morse, 1947; Loomis and Shull, 1937)

Reagents:

Anthrone reagent (0.2% in concentrated H_2SO_4).

Standard glucose solution (10mg/100ml)

Procedure:

About five gms of rootstocks were homogenzed well with 10ml of distilled water. The content was filtered through double layer of muslin cloth. To the filtrate, twice the volume of ethanol was added to precipitate the polysaccharide, mainly the starch. After kept it overnight in cold, the precipitate was collected by centrifugation at 3000g for about 15 min. The precipitate was dried over a steam bath, then 40ml of 1M HCl was added to the dried precipitate and heated to about 70°C. It was then transferred to a volumetric flask and diluted to 100ml with 1M HCl. Diluted solution (1ml) was taken in another 100 ml volumetric flask and made upto 100 ml with 1M HCl.

Aliquots of 1ml of rootstock extract was pipetted into test-tubes in duplicate and treated in the same manner as described for free sugar estimation, previously.

The amount of starch in rootstock was calculated from the standard curve of glucose (Fig. 2.1).

Calculation:

Percentage of starch content (gm per 100 gm of rootstock)

$$= \frac{\text{Amount of starch obtained}}{\text{Weight of rootstock}} \times 100$$

2.2.8. DETERMINATION OF PECTIN CONTENT OF ROOTSTOCK:

Pectin content of rootstock was determined by the King's (1975) gravimetric determination of pectin as calcium pectate.

Reagents:

95% Ethyl alcohol, 0.002M NaOH, 0.1M Acetic acid and 10% $CaCl_2$

Procedure:

Four to six gms of rootstock were cut into small pieces and homogenized well with water and heated on a steam bath for 5-10 min. in a 50ml beaker. Then, added 95% ethyl alcohol with little stirring and incubated the solution for about two hours at 50°C. The extract was filtered through two layers of muslin cloth and re-extracted the ground tissue for three times in hot 95% ethyl alcohol. Then added a small excess of 0.002M NaOH and allowed the solution to stand for one hour. Then, added 20ml of 0.1M acetic acid and 40 ml of 10% $CaCl_2$ solution and incubated for one hour, boiled and filtered through a Whatmannn no. 41 filter paper. Then boiled with 40ml of water and added 20ml of 0.1M acetic acid. The viscous solution was dried and the amount obtained as calcium pectate.

Calculation:

The percentage of pectin as calcium pectate (gm per 100 gms of rootstock)

$$= \frac{\text{Amount of calcium pectate obtained}}{\text{Weight of rootstock}} \times 100$$

2.2.9. DETERMINATION OF CRUDE FIBRE CONTENT OF ROOTSTOCK:

Crude fibre was determined by the following method (AOAC, 1980).

Reagents:

H_2SO_4 (0.26N), NaOH (1.25%), Ethanol and Ether.

Procedure:

Three gms of fat free rootstock were taken into 500ml beaker and 200ml of boiling 0.26N H_2SO_4 was added. The mixture was then boiled for 30 minutes, keeping the volume constant by the addition of water at frequent intervals (a glass rod inserted in the beaker for smooth boiling). At the end of this period, the mixture was filtered through a muslin cloth and the residue was washed with hot water till free from acid.

The extract was boiled for 30 minutes (keeping the volume constant as before) and the mixture was filtered through muslin cloth. The extract was washed with hot water until free from alkali, followed by washing with some ehtanol and ether. It was then transferred to a crucible, dried overnight at 80°C-100°C and weighed.

The crucible was then heated in a muffle furnace at 600°C for three hours, cooled and weighed again. The difference in the weight represented the weight of crude fibre. The percentage of crude fibre (on dry basis) was calculated from the formula given below.

Calculation:

Crude fibre content (gm per 100 gm of rootstock)

$$= \frac{\text{Amount of dried extract}}{\text{Weight of rootstock}} \times 100$$

2.2.10. DETERMINATION OF PROTEIN CONTENT OF ROOTSTOCK:

Protein content of fresh rootstock of the plant was determined by the method of Micro-Kjeldahl (Wong, 1923).

Reagents:

Solid potassium sulphate, Conc. H_2SO_4, 5% $CuSO_4.5H_2O$ in distilled water, 0.01N H_2SO_4, Conc. sodium hydroxide solution (5N approximately) and Few quartz chips.

Boric acid solution containing bromocresol green (receiving fluid): 10 gms of boric acid was dissolved in hot water (250ml) and cooled. 1ml of 0.1% bromocresol green in alcohol was added and diluted to 500ml with distilled water.

Nitrogen determination apparatus according to Paranas-Wagner, made of JENA glass-all connections with inter-changeable ground joints.

Procedure:

a) Digestion:

Conc. H_2SO_4 (4-6ml), 1.0gm K_2SO_4, one to two drops of 5% $CuSO_4$ solution (catalyst) and some quartz chips were added (to avoid bumping) to 1-2 gms of rootstock in a Kjeldhal flask. The mixture was heated till it became light green (2-3 hours)

b) Collection of ammonia:

The digestion was carried out in the steam distillation chamber of the nitrogen determination apparatus. The chamber is designed to act as a Micro-Kjeldhal flask and can be easily detached, when needed. After completion of digestion, the steam distillation chamber containing the digested mixture was filtered back to the nitrogen determination apparatus. Boric acid solution (15ml) taken in a small flask was placed, so that the condenser outlet dipped below the surface of boric acid solution. Sufficient amount of conc. sodium hydroxide solution (approximately. 30-40ml) was added to the digest in the chamber to neutralize the amount of acid present. Steam was generated from the steam-generating flask and the sample in the chamber was steam distilled until 20ml of distillate was collected in boric solution. The condenser outlet was then rinsed with little distilled water and the receiving flask was removed.

c) Estimation of ammonia:

The ammonia in boric acid solution was titrated with 0.01N H_2SO_4 until the solution had been brought back to its original yellow green colour. The titration was repeated with a control containing 20ml boric acid solution and diluted approximately to the final volume of 40ml. The two differences gave the volume of acid required for titration from which the protein contents were estimated.

The total nitrogen was calculated using the formula given below:

1ml of 0.01N H_2SO_4 = 140μg of nitrogen in NH_3

Thus from the volume of standard sulphuric acid (H_2SO_4) used for titration, the amount of nitrogen in sample was calculated. This value multiplied by 6.25 give the approximate protein content of the sample used.

Percentage of protein content of rootstock (gm per 100 gms of rootstock)

$$= \frac{\text{Amount of protein obtained}}{\text{Weight of rootstock}} \times 100$$

2.2.11. DETERMINATION OF CALCIUM CONTENT OF ROOTSTOCK:

Calcium content was determined by titrimetric method (Bernard, 1965).

Reagents:

Hydrochloric acid (conc.), Ammonium oxalate (6%), Methyl red indicator, Dilute sulphuric acid (2N), Strong ammonia and Potassium permanganate (Jam brand, West Germany) solution (N/100).

Preparation of stock solution:

The ash obtained as described earlier was moistened with a small amount of distilled water (0.5-1.0ml) and then 5ml conc. HC1 was added to it.

The mixture was evaporated to dryness on boiling water bath and 5ml of concentrated HC1 was added again and the solution was evaporated to dryness. The residue was dissolved in about 4ml of HC1 and a few ml of water, and the solution was warmed on a boiling water bath. The warmed solution was then filtered using Whatmann no. 40 filter paper. After cooling, the volume was made upto 100ml with distilled water and suitable aliquots were used for the estimation of calcium.

Procedure:

25ml of the stock solution was taken in a conical flask and 125ml of double distilled water was added to it. A few drops of methyl red indicator was added and the mixture was neutralized with ammonia, till the pink colour changed to yellow. The solution was heated to boiling and 10ml of ammonium oxalate was then added. The mixture was allowed to boil for a few minutes and then glacial acetic acid was added to it till the colour became distinctly pink. The mixture was kept in dark at room temperature for an hour. When the precipitate was settled down, the

supernatant was tested with a drop of ammonium oxalate solution to ensure the completion of the precipitation. The precipitate was then filtered through Whatmann no. 40 filter paper and washed with warm water till the precipitate became free of oxalate (tested with $CaC1_2$). The precipitate was transferred to a beaker by piercing a hole in the filter paper and about 5 to 10ml H_2SO_4 (2N) was poured over it. The solution was then heated to about 70°C and titrated with N/100 $KMnO_4$ solution.

Calculation:

1ml of N/100 $KMnO_4$ solution ≡ 0.2004 mg of calcium.

Amount of calcium content (mg per 100 gm of rootstock).

$$= \frac{\text{mg of calcium obtained}}{\text{Weight of rootstock}} \times 100$$

2.2.12. DETERMINATION OF PHOSPHORUS CONTENT OF ROOTSTOCK:

Phosphorus content of root stock was determined by the method of Ranganna (1986).

Preparation of molybdate solution:

12.50gm of sodium molybadte ($Na_2MoO_4.2H_2O$) was dissolved in 10N H_2SO_4 and diluted to 500ml with 10N H_2SO_4.

Preparation of standard phoshate solution:

Exactly 0.219gm of potassium dihydrogen phoshate was dissolved in deionized water and diluted to one litre.Then, 1ml solution ≡ 0.05mg phosphous.

Preparation of stock solution:

Same as described previously in case of calcium.

Suitable aliquots of stock solution was used for the estimation of phosphorus.

Procedure:

One ml of rootstock extract, 2ml of hydrazine sulphate and 5ml of molybdate reagent were taken in a 50ml volumetric flask and made upto the mark with deionized water. The mixture was mixed well. The flask was kept immersed in a boiling water bath for ten min, then it was removed and cooled rapidly. The absorbance of each of the solution was measured at 830 nm against reagent blank.

A calibration curve was constructed in the usual process by using six standard phosphorus solution containing 1, 2, 3, 4, 5 and 6 ppm of phosphorus. The mg percentage of phosphorus present in rootstock extract was calculated by using the standard curve of phosphorus (Fig. 2.3).

Calculation:

Amount of phosphorus present (mg per 100gm of rootstock)

$$= \frac{\text{mg of phosphorus obtained}}{\text{Weight of rootstock}} \times 100$$

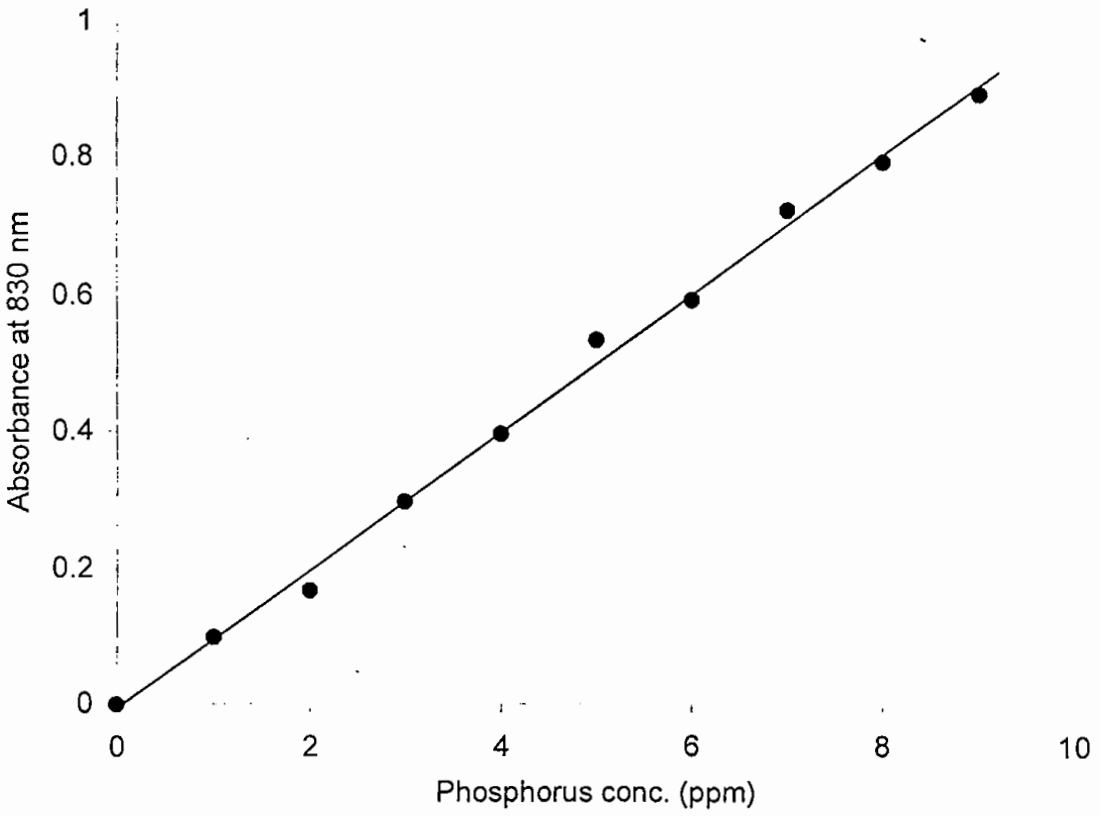


Fig. 2.3. Standard curve for estimation of phosphorus.

2.2.13. DETERMINATION OF IRON CONTENT OF ROOTSTOCK:

Iron content of root stock was determined following the method as described by Ranganna (1986).

Reagents:

a) 4N HC1

b) Potassium thiocyanate solution (20%)

c) Preparation of Fe (III) standard solution:

Exactly 0.702gm of ferrous ammonium sulphate was taken in a 1 litre volumetric flask and dissolved in 100ml (2gm/l.) distilled water. A dilute solution of potassium permanganate was run slowly until a faint pink colouration remained after stirring well. The resulting solution was then made upto mark with distilled water.

Then, 1ml solution contains ≡ 0.1mg of Fe (III).

Procedure:

10ml of rootstock extract was taken in 50ml volumetric flask; 2 ml of 4N HC1 and 5ml of 20% potassium thiocyanate solution were added. Then the flask was made upto the mark with de-ionized water. The experiment was done three times. The absorbance for each of the solution was measured at 480 nm against a reagent blank. Iron content of the solution was determined by constructing a standard curve. A standard curve of iron was prepared by taking 5ml, 10ml, 15ml, 20ml, 25ml and 30ml of the standard (0.1mg/ml) ammonium iron (III) sulphate solution. To the flask, 2ml of 4N hydrochloric acid and 5ml of 20% potassium thiocyanate solution were added and diluted upto the mark and shaken slowly for uniform mixing. The absorbance for each of the solution was measured at 480 nm against a reagent blank.

The mg percent of iron present in rootstock extract was calculated by using the standard curve (Fig. 2.4).

Calculation:

Amount of iron content (mg per 100gm of rootstock)

$$= \frac{\text{mg of iron obtained}}{\text{Weight of rootstock}} \times 100$$

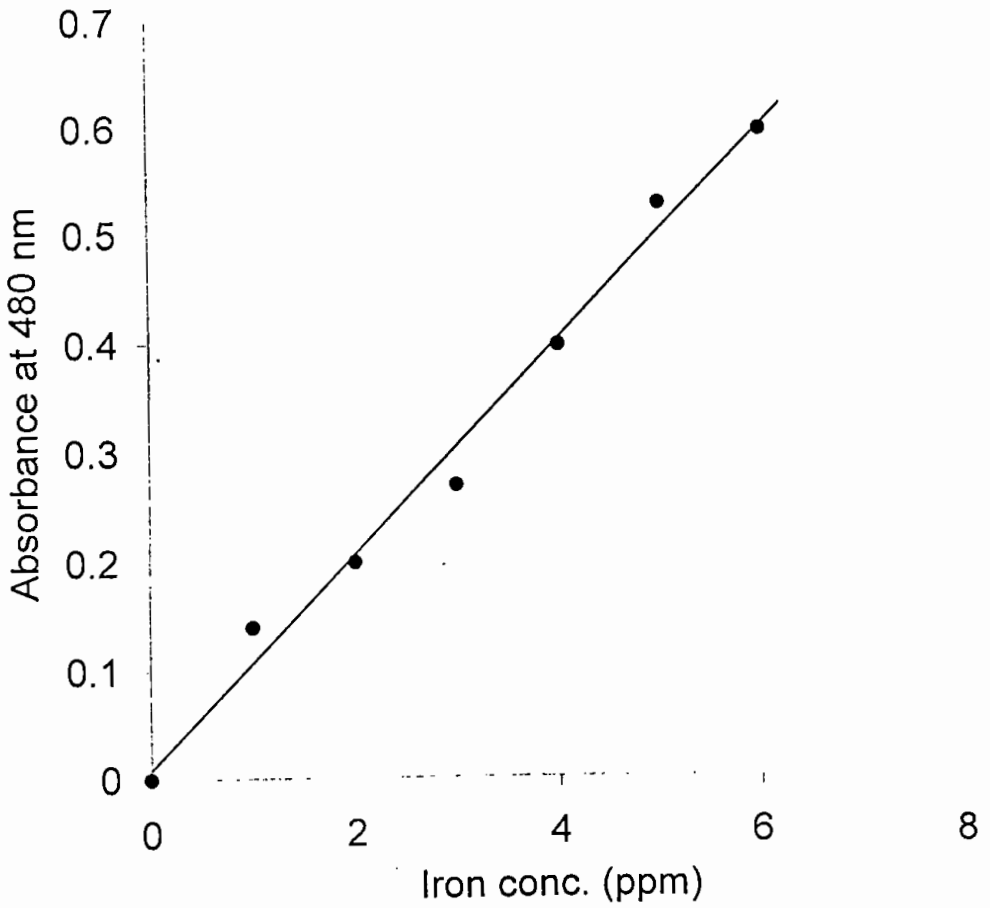


Fig. 2.4. Standard curve for estimation of iron.

2.2.14. DETERMINATION OF VITAMIN-C CONTENT OF ROOTSTOCK:

Vitamin-C content of rootstock was determined by the Bessey's titrimetric method (1933).

Reagents:

Dye solution: 200mg of 2,6 dichlorophenol indophenol and 210mg of sodium bicarbonate were dissolved in distilled water and made upto 1000 ml. The solution was filtered.

3% metaphosphoric acid reagent: 3 gms of metaphosphoric acid was dissolved in 80ml of acetic acid and made upto 100ml with distilled water.

Standard Vitamin-C solution (0.1mg/ml): 10mg of pure vitamin-C was dissolved in 3% metaphosphoric acid and made upto 100ml with 3% metaphosphoric acid.

Method:

Standard vitamin-C solution (10 ml) was taken in a conical flask and titrated it with the dye solution.

Four to six gms of rootstocks were cut into small pieces and homogenized well with 3% metaphosphoric acid (approximately 20ml) and filtered it through double layer of muslin cloth. The filtrate was centrifuged at 3,000g for 10 minutes and the clear supernatant was titrated with 2,6 dichlorophenol indophenol solution. The amount of vitamin-C present in the extract was determined by comparing with the titration result of standard vitamin-C solution.

Calculation:

Percentage of vitamin-C content (mg per 100 gms of rootstock)

$$= \frac{\text{mg of vitamin-C obtained}}{\text{Weight of rootstock}} \times 100$$

2.2.15. DETERMINATION OF VITAMIN-B_1 CONTENT OF ROOTSTOCK:

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

Reagents:

Potassium ferricyanide (2%)

Oxidizing reagents : 10ml of 2% potassium ferricyanide was mixed with 3.5 N NaOH solution (90ml). The solution might be used within 4 hours.

Quinine sulphate, 0.2N HC1 and Alcohol

Standard thiamine hydrochloride preparation:

About 25mg of thiamine hydrochloride was transferred in 100ml volumetric flask and it was dissolved in 30ml of dilute alcohol solution and made upto the mark. The pH was adjusted to 4.0 with dilute HC1 and stored in a light resistant container.

Procedure:

Five to six gms of fresh rootstocks were cut into small pieces and homogenized with 0.2N HC1. The mixture was heated on a steam bath and then cooled.

5ml of standard thiamine-HC1 solution was mixed rapidly with 3ml oxidizing reagent and 20ml of isobutyl alcohol was added within 30 seconds, then mixed the mixture vigorously for 90 seconds by shaking the tubes manually. A blank was prepared only by substituting the oxidizing reagent with an equal volume of 3.5N sodium hydroxide and proceeded in the same manner. 2ml of dehydrate alcohol was added, swirled for few seconds, allowed the phase to be separated and decanted or drawn off and transferred into cuvettes, then measured the

fluorescence. Rootstock extracts (5ml) were pipetted in different test tubes and treated in the same manner as described above.

Calculation:

The amount (in mg) of thiamine hydrochloride in each 5ml of the rootstock extracts was calculated from the formula (A-b) / (S-d), in which A and S were the average fluorometer reading of the rootstock extract and standard preparation with oxidizing reagent, respectively, and b and d were the readings for the blanks of rootstock extract and standard preparation, respectively.

mg percentage of vitamin-B_1 (mg per 100gm of fresh rootstock)

$$= \frac{\text{mg of vitamin-B}_1 \text{ obtained}}{\text{Weight of rootstock}} \times 100$$

2.2.16. DETERMINATION OF VITAMIN-B_2 CONTENT OF ROOTSTOCK:

Vitamin B_2 content of rootstock was determined by the method of Anon (1965).

Reagents:

0.02N acetic acid, 0.1N H_2SO_4, 0.1N NaOH, 0. 1N HCl, 4% Potassium permanganate and Hydrogen peroxide

Procedure:

Standard preparation: 50mg of riboflavin was mixed with 300ml of 0.02N acetic acid and the mixture was heated on a steam bath, with frequent agitation until the riboflavin was dissolved. Then the solution was cooled and made upto 500ml with 0.02N acetic acid. This solution was diluted appropriately with 0.02N acetic acid to made final riboflavin concentration of 10μg/ml.

Extraction of riboflavin from rootstock:

Fresh rootstock (5-6 gms) were cut into small pieces and homogenized well with 0.1N H_2SO_4 (about 50ml). The mixture was heated in an autoclave at 121-123°C for 30 minutes, then cooled it and filtered through double layer of muslin cloth. The filtrate was made upto 100ml with distilled water and 25ml of this solution was taken in a beaker and 25ml of water was added to it. The mixture was agitated vigorously and adjusted the pH to 6.0 - 6.5 with 0. 1N NaOH. Immediately, 0.1N HC1 was added until no precipitation occurs. The extract was again filtered and pH of the extract was adjusted to 6.6 - 6.8 with 0.1N NaOH.

10ml of rootstock extract was taken in the test tube, 1.0 ml of water and 1.0ml of glacial acetic acid were added to it. The mixture was then mixed with 0.05ml of potassium permanganate solution and allowed to stand for two minutes, then 0.5ml of hydrogen peroxide solution was added, where upon the permanganate color was destroyed within 10 seconds. The tube was shaken vigorously until excess oxygen expelled, then 1ml of standard solution was pipetted in a test tube and treated in the same manner as that described for the rootstock extract. In a suitable fluorometer, the fluorescence of the tubes was measured. Then to each tubes, 20 mg of sodium hydrosulphite were added, mixed well and measured the fluorescence, within 5 seconds.

Calculation:

The quantity in mg in each ml of the rootstock extract was calculated by the formula, $0.0001\ (I_u-I_B)\ (I_S-I_u)$

Where,

I_u = Average reading for rootstock extract

I_S = Average reading for standard preparation

I_B = Average reading after mixed with sodium hydrosulphite

mg% of vitamin-B_2 content in rootstock (mg per 100gm of rootstock)

$$= \frac{\text{mg of vitamin-}B_2\text{ obtained}}{\text{Weight of rootstock}} \times 100$$

2.3. ANALYSIS OF ROOTSTOCK OIL:

2.3.1. EXTRACTION OF OIL FROM THE ROOTSTOCK:

The oil was extracted from the rootstock by solvent extraction process (South comb, J,E; 1926).

Principle:

Extraction of oil in a sutiable apparatus with an adequate solvent, petroleum ether (40-60°C)

Apparatus:

1.Electrical weighing balance (Mettler H 18), 2.Mortar and pestle, 3. Soxhlet apparatus and 4. Rotary evaporator.

Chemicals:

1. Petroleum ether (40-60^{0}C), analytical reagent grade.

Procedure:

About 150gm of rootstock was taken in mortar and crushed well to almost powder form by a hand pestle. The rootstock powder was placed in the extractor of soxhlet apparatus and the oil was extracted by passing petroleum ether vapor at 50-60°C through a pipe attached to the extractor (beginning from lower part and ending at the top of the extractor). After condensing the petroleum ether together with oil

comes back through the lower part of the extractor to the evaporator and the process was cyclic. The extraction of oil was completed in eight cycles. The petroleum ether extract so obtained was evaporated under reduced pressure to obtain oil.

2.4. PURIFICATION OF THE CRUDE OIL:

Materials and reagents:

1. Separating funnel.
2. Diethyl either.
3. Saturated sodium chloride solution.

Procedure:

The oil obtained from the rootstock by solvent extraction process was subjected to the following treatment for purification (Haque, M.E., 1975).

The rootstock oil (100 gm) was taken in a separating funnel and mixed with water (100 ml), ether (200 ml) and saturated sodium chloride solution (25 ml). The content of the separating funnel was shaken well and allowed to stand for sometimes when two distinct layers were separated. Discarding the aqueous layer, the organic layer was again mixed with 100 ml of distilled water and saturated solution of sodium chloride (25 ml). Twenty minutes after, organic layer was separated from the aqueous layer and was subjected same as before, to once more. Finally, the ethereal extract was taken in a conical flask and dried over anhydrous sodium sulfate. The dried ethereal solution was then evaporated at 40°C to give the purified oil.

2.5. CHARACTERIZATION OF ROOTSTOCK OIL:

2.5.1. STUDY OF THE PHYSICAL CHARACTERTICS OF OIL:

2.5.1.1. Determination of specific gravity of oil:

The specific gravity (Sp. Gr.). of oil was determined by means of a specific gravity bottle (Hilditch, T.P., 1947).

Procedure:

The specific gravity bottle was cleaned, dried and weighed. The bottle was then filled with distilled water which was previously boiled and cooled at room temperature by holding it in an inclined position to avoid the formation of bubbles. It was then immersed in a constant temperature water bath at 20°C with its stoppered end just above the level of the bath for half an hour. The bottle was then removed from the bath, wiped, dried with tissue paper and allowed to stand for 15 minutes and weighed.

The procedure was repeated by replacing water with oil. The specific gravity was calculated using the following formula.

$$\text{Specific Gravity} = \frac{\text{Weight of the oil in bottle}}{\text{Weight of the distilled water in bottle}} \times 100$$

2.5.1.2. Determination of the Refractive index of oil:

Refractive index (R.I.) of a medium is the ratio of the speed of light at a definite wavelength in vacuum to its speed in the media. The refractive index is determined (Hilditch, T.P., 1949) by using Abbe type refractometer.

Principle:

Measurement by means of a suitable refractometer of the liquid sample.

Apparatus:

1. Abbe Refractometer (standard model 60/70) and 2.Thermostat.

Procedure:

The hinged prisms of refractometer were opened and cleaned with a little ether on a tissue paper. Two or three drops of oil was placed on the face of the prism. The prism was then closed and the apparatus left for 2 or 3 min to attain euilibrium temperature. The reading was directly measured by rotating the focusing telescope until the line of total reflection passed through the intersection of the two hair lines fixed in the field of view.

2.5.1.3. Determination of smoke point, flash point and fire point of the oil:

The smoke point, flash point and fire point were determined according to the Official Methods of the American Oil Chemists Society (AOCS).

2.5.1.4. Determination of the Pour point, Cloud point, and Solidification point of the oil

The pour, clound and silidification points of the oil were measured according to the ASTM standard method of the lubricating oil.

2.5.2. STUDY OF THE CHEMICAL CHARACTERISTICS OF THE OIL:

2.5.2.1. Determination of saponification value and saponification equivalent Principle:

Boiling of the test portion under reflux with ethanolic potassium hydroxide solution and titration of the excess potassium hydroxide with hydrochloric acid in presence of indicator.

Apparatus:

1. Round bottom flask, 2. Reflux condenser, 3. Pipette and burette, 4. Water bath.

Reagents:

1. Alcoholic solution of potassium hydroxide (0.5 N approximately).

2. 0.5 N hydrochloric acid

3. Phenolphthalein solution (1% in alcohol).

Procedure:

The oil (1-2 gm) was taken in a conical flask and 25 ml of alcoholic potassium hydroxide solution was added to it. The flask was then connected to a reflux condenser and heated on boiling water bath so that the alcoholic solution boiled gently for 30 minutes. During this time the flask with its content was shaken occasionally to prevent agitation, A blank experiment (without oil) was performed simultaneously in the same manner as described. After 30 minutes, both the flasks were removed from the water bath and their contents while still hot, were titrated with hydrochloric acid (0.5 N) using phenolphthalein as indicator.

The saponification value (IUPAC, 1979) and saponification equivalent (S.E) were calculated using the following formula.

$$S.V. = \frac{56.1 \times (A-B) \times \text{Strength of acid used}}{W} \times 100$$

Where, A = ml of acid required for the blank experiment.

B = ml of acid required for the text experiment.

W = Weight of the oil taken in gm.

S.V. = Saponification value.

$$S.E. = \frac{561000}{\text{Saponification Value of Oil}}$$

2.5.2.2. Determination of Acid value and percentage of free fatty acid (as oleic).

Acid value is the mg of KOH required to neutralize the free fatty acids present in one gm of oil. This is used for determining the rancidity due to free fatty acids.

Acid value (A.V.) of the oil was determined following the procedure as reported earlier (Williams. K. A., 1966).

Principle:

Solution of a known quantity of the oil to be analyzed in a mixture of ethanol and diethyl ether followed by titration of the free fatty acids present with an ethanolic solution of KOH.

Apparatus:

1. Conical flask.
2. Burette and pipette.

Reagents:

1. Solvent mixture of ethanol and diethyl ether, (1:1, v/v of 95%). Neutralized exactly just before use, by means of the KOH solution in presence of 0.3 ml of phenolphthalein solution per 100 ml of the mixture.
2. Potassium hydroxide solution (0.1 N) in ethanol, accurately standardized.
3. Phenolphthalein solution, 1% in 95% ethanol.

Procedure:

A known amount of oil (3-5 gm) was taken in a conical flask and mixed with 50 ml of 95%alcohol. The mixture was heated to boiling and the content of the flask

was titrated with alcoholic KOH (0.1N) until a faint pink color persisted for atleast 10 seconds. The content of the flask was shaken continuously and vigorously during the titration. Acid value of the oil was calculated using the formula given below.

$$A.V.= \frac{56.1 \times N \times V}{W}$$

Where,

A.V. = acid value, N = Strength of alklali, V = Volume of alcoholic alkali required for titration and W = Weight of the oil taken in gm.

2.5.2.3. Determination of percentage of free fatty acid:

The percentage of free fatty acid (%FFA) was measured using the following formula.

$$\%FFA = \frac{AV \times M}{56.1}$$

2.5.2.4. Determination of Iodine value:

Iodine value of the oil was determined by the Hanus method (Williams, K.A 1966)

Principle:

Addition to the test portion of an iodine solution, after a time of reaction, determination of the excess halogen by addition of potassium iodide aqueous solution and titration of the liberated iodine with a standardized sodium thiosulphate solution.

Apparatus:

1. Wide necked glass bottle with ground glass stopper, 2. Burette and 3. Pipette

Reagents:

1. Chloroform (E. merck).

2. Potassium iodide (15% solution in distilled water).

3. Starch indicator: Starch (1 gm) was dissolved in 50 ml hot water and diluted to 100 ml with cold water.

4. 0.1 N sodium thiosulphate solution.

5. 0.1N potassium dichromate solution.

6. Conc HCl.

7. Sodium bicaronate.

8. Hanus solution

Preparation of Hanus solution:

Exactly 13.2 gm of iodine was dissolved in 12 ml of glacial acetic acid and it was warmed to dissolve the iodine completely. The mixture was cooled at room temperature (25°C) and diluted with glacial acid upto 1 liter. Finally enough bromine was added to double the halogen content, about 3 ml was sufficient.

Procedure:

The oil (2gm) was dissolved in 10 ml chloroform in a dry glass stoppered bottle (500ml). To the content, 25ml of Hanus solution was added and the mixture was allowed to stand in the dark for exactly 30 min with occasional shaking. Potassium iodide solution (10ml) was mixed to it and the mixture was shaken well. Freshly boiled cool water (100ml) was added to the mixture and the content of the boiled cool water (100ml) was added to the mixture and the content of the bottle was titrated with sodium thiosulphate solution, using starch solution as indicator.

A blank experiment was performed exactly in the same manner without oil. The iodine value was calculated using the following formula:

$$\text{I.V.} = \frac{S \times (X-Y) \times 0.127}{W} \times 100$$

Where,

S= Strength of the sodium thiosulphate solution

X= ml of sodium thiosulphate solution required in blank test.

Y = ml of sodium thiosulphate solution required in the experiment.

W= Weight of the oil taken in gms.

2.6. RESULTS AND DISCUSSIONS:

2.6.1. MOISTURE CONTENT OF ROOTSTOCK:

Moisture plays an important part in the growth activities of plants, herbs etc. Water is indispensable to the absorption and transportation of food to carry on photosynthesis, metabolism of materials and the regulation of temperature. Moisture is also essential for most of the physiological reactions in plant tissue and in its absence, life does not exist (Rangaswami, 1976). Moisture content of rootstock was found to be about 62.8%.

2.6.2. ASH CONTENT OF ROOTSTOCK:

Most of the inorganic constituent or minerals and its ash contained in the rootstock of *Potamogeton nodosus* Poir was calculated to be 2.13%.

2.6.3. LIPID CONTENT OF ROOTSTOCK:

Lipid content of the rootstock was measured to be 32.74mg%. It can be concluded that the rootstock contained lower amount of lipid than seeds or fruits.

2.6.4. TOTAL SUGAR, REDUCING AND NON-REDUCING SUGAR CONTENT OF ROOTSTOCK:

Rootstock of *Potamogeton nodosus* Poir contains both reducing and non-reducing sugar. Among fuel materials the amount of sugar content was in second position i.e. it was less than that of protein but greater than the amount of total lipid. The total sugar content was 2.78%, of which reducing sugar was 0.53% and non-reducing sugar was 2.25%.

2.6.5. STARCH CONTENT OF THE ROOTSTOCK:

Starch is the storage carbohydrate of chlorophyll containing plants. In plants, the starch is laid down in the cells in granules.

Starch (polysaccharide) content of the rootstock was found to be 5.56%.

2.6.6. PECTIN CONTENT OF ROOTSTOCK:

Pectic substances are found in primary cell walls and in intercellular cement. The simplest monomer of pectin is galacturonic acid. A simplest classification of pectic substances is pectic acid, pectin and protopectin. The pectic substances are measured as calcium pectate. The calcium pectate present in the rootstock was determined to about 7.27%.

2.6.7. CRUDE FIBRE CONTENT OF ROOTSTOCK:

Cellulose, lignin and pentosan are the components of crude fibre (Anon, 1975). Crude fibre has pronounced effect on the digestion and absorption process of nutrients. Crude fibre content of the rootstock was estimated to be about 1.02%.

2.6.8. PROTEIN CONTENT OF ROOTSTOCK:

Protein content of plants and herbs are of primary importance not only as component of nuclear and cytoplasmic structures, but also including, as they must be the full complement of enzymes involved in metabolism during growth, development, maturation and the post harvest of the plant (Hansen, 1970).

The rootstock of *Potamogeton nodosus* Poir contains considerably significant amount of protein and it is about 14.82%.

2.6.9. CALCIUM CONTENT OF ROOTSTOCK:

Calcium is a constituent of cell walls. It is needed in large amount for cell division of the growing part of the plant. In deficiency of calcium, plant root tent to be short and stubby as a result leading to the death of their tissues (Rangaswami, 1976). Calcium is also required for many essential enzyme activities in leaves.

Calcium content of the rootstock as measured was about 149mg%.

2.6.10. PHOSPHORUS CONTENT OF THE ROOTSTOCK:

Phosphorus is one of the major nutrients for the growth of the plant. It is the structural constituent of nucleotide (ATP), which is an energy carrier for all the metabolic activities. It is also essential for constituent of cell nucleus, cell division and development of meristematic tissues (A.F. Chandrnath et al., 1991).

Phosphorus content of the rootstock was about 112 mg%.

2.6.11. IRON CONTENT OF ROOTSTOCK:

Iron plays important role in maintaining cell permeability in cell division and in metabolic activities of the rootstock. Iron content of the rootstock was fairly greater than other minerals and it was measured to be 226mg%.

2.6.12. VITAMIN-C, VITAMIN-B_1 AND VITAMIN-B_2 CONTENT OF THE ROOTSTOCK:

Vitamin-C plays an important role in the metabolism of plants. It occurs as L-ascorbic acid and in its oxidized form as dehydroascorbic acid in nearly all the plants. Ascorbic acid reduced quinones to phenols and this reaction has received much attention in defense mechanism.

Thiamine exists in tissues mostly in the form of thiamine pyrophosphate known as co-carboxylase. TPP serves as co-enzyme in the metabolism of carbohydrate, fat and protein. Beside the metabolic role, thiamine has a specific role in neurophysiology, dependent of its co-enzyme function. In plants and herbs, it is very essential. Thiamine is also essential for growth, normal appetite, digestion and healthy nerves.

Vitamin-B_2 combines in the tissue phosphoric acid to become part of the structure of two flavin co-enzymes, FMN and FAD, acts on the activities of the enzymes. It is essential for normal growth and tissue maintenance.

The vitamins content of the rootstock were found to be 0.08mg%, 12.86mg% and 16.23mg% for Vitamin-C, Vitamin-B_1 and Vitamin-B_2 respectively.

Table 2.1 : Nutrient compositions of the rootstock of *Potamogeton nodosus* Poir.

Name of the composition	**Amount**
Moisture	68.2 ± 0.01%
Ash	2.13 ± 0.03%
Lipid	32.74 ± 0.12 mg%
Total Sugar	2.78 ± 0.02%
Reducing Sugar	0.53 ± 0.02%
Non Reducing Sugar	2.25 ± 0.03%
Starch	5.56 ± 0.04%
Pectin as Ca-Pectate	7.27 ± 0.07%
Fiber	1.02 ± 0.01%
Protein	14.82 ± 0.14%
Calcium	149 ±0.03 mg%
Phosphorus	112 ±0.04 mg%
Iron	226 ±0.16 mg%
Vitamin-C	0.08 ± 0.04 mg%
Vitamin-B_1	12.86 ±0.03 mg%
Vitamin-B_2	16.23 ±0.05 mg%

2.7. ANALYSIS OF THE ROOTSTOCK OIL:

2.7.1. CHARACTERIZATION OF OILS:

The physical and chemical properties of oil and fats vary between certain limits and due to this small variations, they are considered to be constants. Although the chemical constants are more important to characterize an oil, but the physical constants are also often capable of giving valuable information. The important chemical constants are iodine value, saponification value, acid value, % of free fatty acids etc, whereas specific gravity, refractive index, etc are the important physical constants of oil. Biological properties also provide some valuable information about medicinal value.

The physical and chemical properties of rootstock oil were analyzed and the results are presented in Table-2.2 and Table-2.3 respectively.

2.7.1.1. Specific Gravity:

The specific gravity of fat or oil does not vary as a general rule to an extent which makes this property useful in discriminating between one fat and another. The specific gravity of practically all fats or oils lies between 0.90 and 0.95 (Hilditch, P., 1949).

The specific gravity of *Potamogeton nodosus* rootstock oil was found to be 0.920 at 25°C.

2.7.1.2. Refractive index:

The refractive power of oils or fats varies somewhat widely but chiefly governed by the proportion and degree of unsaturation present.

As shown in the Table-2.2, the refractive index of rootstock oil was found to be 1.4710 at 28°C. The refractive index of blackcurrant seed oil is 1.4770-1.4830 (http://www.greencottage.com/oils/blackcurrent.html).

The result suggested that the rootstock seed oil contained fairly large amount of unsaturated fatty acids.

2.7.1.3. Smoke Point, flash Point and Fire Point:

Thermal properties, i.e. the smoke point, flash point and fire point of rootstock oil were found to be 218°C, 322°C and 356°C respectively.

2.7.1.4. Pour point, Cloud Point and Solidification Point:

The pour point, cloud point and solidification point of rootstock oils were found to be - 6.4°C, 2.1°C and - 8.4°C respectively.

Table-2.2: Physical constants of *Potamogeton nodosus* Poir (Pondweed) oil.

Constants	***Potamogeton nodosus* (Pondweed) oil**
Specific gravity at 25^0C	0.920
Refractive index at 25^0C	1.4710
Smoke point (^{0}C)	218
Flash point (^{0}C)	322
Fire point (^{0}C)	356
Cloud point (^{0}C)	2.1
Solidification point (^{0}C)	-8.4
Pour point (^{0}C)	-6.4

2.7.1.5. Saponification Value and Saponification Equivalent:

As shown in Table-2.3 the saponification value of the rootstock oil was 178.27 while the saponification equivalent of the rootstock oil was 312.26 as calculated from the saponification value.

Fats or oils consisting of mainly C_{18} fatty acids along with some palmitic acid, a little unsaponificable matter and trace of free fatty acid generally have saponification equivalent around 290.80 indicating the presence of appreciable quanity of higher fatty acids (Ackmam, 1966).

It is clearly indicated from the results that the experimental rootstock oils contained mainly fatty acids of C_{18} molecular weight along with some palmitic acid.

2.7.1.6. Acid Value and Percentage of Free Fatty Acid:

The acid value and percentage of free fatty acid of rootstock oil was found to be 5.675 and 2.058 respectively.

A high percentage of free fatty acid (above 1.15%) is a determination or indication of unsuitability of the oil for edible purposes (Jaimeson, 1932).

Although the percentage of free fatty acid content of rootstock oil is slightly higher than the accepted value but it may consider to use as edible oil as it contained higher amount of unsaturated fatly acids.

2.7.1.7. Iodine Value

It was found in the present study that the iodine value of rootstock oil was between 112-120.

Table-2.3: Chemical characteristics of *Potamogeton nodosus* Poir (Pondweed) oil.

Characteristics	***Potamogeton nodosus* (Pondweed) oil**
Saponification Value	178.27
Saponification Equivalent	312.26
Acid Value	5.675
% of Free Fatty Acids	2.058
Iodine Value	112-120

2.8. DISCUSSIONS

Rootstock of Potamogeton nodosus Poir contains sugar both reducing and non-reducing, vitamins, minerals, trace amount of lipid and a greater amount of protein. Of the major energy producing nutrients, the rootstock contains higher amount of protein. The moisture content was examined to be 68.2% while ash and crude fiber content were 2.13% and 1.02% respectively. Protein content was found to be 14.82% while lipid content was very little. Among fuel materials the amount of sugar content was in second position. The starch (polysaccharide) content was found to be as 5.56%. Among minerals present in the rootstock, this investigation included only calcium, iron and Phosphorus. The results showed that the amount of iron was larger than that of calcium and phosphorus. Vitamin-C content was less than vitamin-B_1 and Vitamin-B_2 content and that was 0.08 mg%, 12.86 mg% and 16.23 mg% respectively for vitamin-C, vitamin-B_1 and vitamin-B_2. The analytical values of various nutrient compositions of the rootstock of *Potamogeton nodosus* Poir are given in table-2.1. On the basis of nutrient contents rootstock might be considered as nutritionally good food quality since it contained significant amount of protein and carbohydrate. Further, rootstock powder may consider to use as a supplementary protein source in food industry after removing the toxic substance, if any present. Although root stock is not a good source of oil but it was characterized with respect to its physical and chemical properties.

The physical properties of rootstock oil such as specific gravity, refractive index, smoke point, flash point, fire point, cloud point, solidification point and pour point were 0.920 at 25°C, 1.4710 at 28°C, 218°C, 322°C, 356°C, 2.1°C, -8.4°C and -6.4°C respectively. These values of physical constants are very much similar to those reported for rice bran oil (M.M. Rahman *et al.*, 1998).

The chemical properties of the rootstock oil such as saponification value, saponificatia equivalent, acid value, % of free fatty acids, and iodine value were 178.27, 312.26, 5.675, 2.058 and 112-120 respectively. The chemical properties of the Aush variety of rice bran oil such as saponification value and iodine value were also found to be similar.

Although rootstock of *Potamogeton nodosus* contains very small amount of oil but the oils might be of good quality due to the presence of unsaturated fatty acids since the iodine value of oil was found to be higher. The edibility of the oil is not so much good as its acid value is higher than five which may be due to action of lipases present in it.

CHAPTER THREE

Purification and Characterization of *Potamogeton nodosus* Rootstocks Lectins

3.1. INTRODUCTION:

Lectins isolated chiefly from plants bacteria, fungi, invertebrates and vertebrates are non - immunoglobulin type carbohydrate recognition molecules. Plant lectins isolated from wide varieties of plants have recently attracted great interest because of their remarkable biological effects that are mentioned in general introduction. Lectins are being also used increasingly to probe the structure of carbohydrate on the surfaces of the normal and malignant cells. Many authors have described the purification, chemical and biological properties of lectins from many kinds of plants. Rootstocks of *Potamogeton nodosus are* good sources of protein and till now no detailed work has done been on the nature of protein present in it. This study describes the purification and characterization of six lectins from rootstock of *Potamogeton nodosus*.

3.2. COLLECTION OF ROOTSTOCKS OF *Potamogeton nodosus* Poir.:

During the month of November to February , rootstocks of *Potamogeton nodosus* was collected from marshy lands of Mohonpur in Rajshahi district. After collection, the rootstocks of *Potamogeton nodosus* were cleaned , dried in the sun and were preserved in the refrigerator at 4^0C.

3.3. METHODS:

3.3.1. PREPARATION OF FAT-FREE MEALS:

In order to purify protein from the rootstocks of *Potamogeton nodosus* in biologically active form , all the operations were performed at 4^0C. First the dried rootstocks were taken in a mortar and pounded uniformly into fine powder. To these powder pre-cooled petroleum ether (40-60°C) was added and homogenized uniformly with a homogenizer. The desired temperature was

maintained by putting ice in the outer chamber of the homogenizer. The oily extract was then kept in a beaker at 4°C for an hour with occasional stirring. The homogenate was then filtered through a clean muslin cloth. The process was repeated again by adding pre-cooled petroleum ether in order to make the homogenate quite fat-free. The filtrate was then further clarified by centrifugation at $8x10^3$ g for 10 minutes. The precipitate obtained after centrifugation were collected, air dried at room temperature and used for extraction of protein.

3.3.2. CHOICE OF EXTRACTION MEDIA:

Proteins from fat free rootstock meals were extracted under identical conditions using five different extracting solvents. After extraction, the total concentration of the protein was determined by measuring the absorbance at 280 nm. The suitable extracting solvent was selected from their ratio of absorbance at 280 nm and 260 nm as reported by Clark and Switzer (1977).

Table 3.1. Preparation of crude protein extract from *Potamogeton nodosus* rootstocks in different extracting solvents.

Extracting Media	Amount of meal (gm)	O.D. at 280 nm	O.D. at 260 nm	Ratio of O.D. 280 & 260 nm
1% CH_3COOH	0.5gm	1.525	1.525	1
Distilled water	0.5gm	1.53	1.55	0.987
Phosphate buffer, pH 7.6	0.5gm	1.53	1.54	0.993
Tris-HCl buffer, pH 8.4	0.5gm	1.52	1.545	0.983
5 Distilled water containing 16 0.15M NaCl pH 5.4	0.5gm	1.54	1.542	0.998

1% CH_3COOH was used as extracting solvent for extraction of crude protein from the rootstocks of *Potamogeton nodosus* as the highest ratio of absorbance (at 280 nm & 260 nm) was found in this solvent.

3.3.3. PREPARATION OF CRUDE PROTEIN EXTRACT:

The fat-free dry powder was mixed uniformly with precooled 1% CH_3COOH in a beaker and kept overnight at 4°C with occasional stirring . The suspension was filtered through a muslin cloth in the cold room .Then the filtrate was collected and clarified further by centrifugation at $8X10^3$ g , 4°C for 20 minutes. The clear supernatant was collected and adjusted to 100% saturation by adding solid ammonium sulfate. The ammonium sulfate precipitate was then centrifuged again at 8×10^3 g, 4^0C for 20 minutes. The precipitate was collected, dissolved in minimum volume of pre-cooled deionized water and dialyzed against cold distilled water for 12 hours with three changes and against 10 mM Tris-HCl buffer, pH 8.4 for 24 hours at 4°C. After centrifugation the clear supernatant was used as crude protein extract and preserved in the deep freeze for experimental purposes.

3.4. PURIFICATION OF PROTEIN

3.4.1. GEL FILTRATION:

i) Activation of gel powder: Sephadex G-50 powder was suspended in 10% acetic acid containing 1M sodium chloride (1 mole of NaCl was dissolved in one liter of 10% acetic acid) in a beaker and left it to swell for overnight. It was stirred by glass rod after short intervals to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water for several times until its pH reached near to neutrality.

ii) Packing of the column: This is very critical step in all types of column chromatographic experiment. If the column is not packed properly, accurate results can never be expected. Because a poorly packed column gives rise to uneven flow rates. The gel suspension was taken in a filtering flask and deaerated by vacuum pump; otherwise it would affect the flow rate of the column after packing. The gel suspension was adjusted so that it was a fairly thick slurry, but not thick enough to retain bubbles. The column was mounted on a stable laboratory stands and its narrow end was fitted with an outlet tube. It was ensured that there was no air bubble in the dead space of the bed support. This was easily achieved by filling approximately 1/4 th of the column, including the outlet tube with distilled water. When dead space was properly filled, the outlet tube was closed with pinch cork and the gel suspension from a gel reservoir was added gently to the column. In order to avoid trapping of any bubble, this was performed by pouring the gel in inner wall of the column. In this way, a column of desired length was packed uniformly with the gel suspension.

iii) Equilibration of the column : After completion of the column packing it was equilibrated with the eluant buffer (10 mM Tris-HCl buffer , pH 8.0). The buffer was continued to run through the column until the pH of the eluate became same as the pH of the eluant buffer.

iv) Application of sample: Before loading of the sample, the outlet tube of the column was opened and the eluant buffer from the top of the gel bed was allowed to diffuse into the gel. The crude extract (3-4 ml) was then loaded on the top of bed . After diffusion of the sample , about 1 ml of eluant buffer was poured on the top of the gel bed and was allowed to diffuse. Then an additional amount of buffer was added, so that the space about 3-4 cm above the gel bed was filled with eluant. The buffer was then allowed to flow continuously through the column at a flow rate of about 24 ml per hour and 3 ml fractions of the eluate were collected by an automatic fraction collector. Absorbance of each fraction was measured at 280 nm .

3.4.2. DEAE-CELLULOSE COLUMN CHROMATOGRAPHY:

A. Procedure:

i) Activation of DEAE-cellulose Powder : The DEAE- cellulose powder was suspended in 0.2M NaOH in a beaker and left it to swell for few hours. During swelling it was stirred gently at short intervals to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water several times until its pH reached near to neutrality. The gel suspension was then transferred to another beaker containing 0.2M HCl and left for few hours. It was again washed with distilled water to neutralize its pH.

ii) Packing of the column : The activated DEAE-cellulose suspension was taken in a filtering flask and deaerated by vacuum pump . A column of desired length was packed with the activated column material. Precaution was taken to avoid trapping of air bubbles during packing.

iii) Equilibration of the column : After packing , the column was equilibrated with 10 mM Tris-HCl buffer , pH 8.4.

iv) Preparation and application of sample : The fraction obtained by gel filtration was dialyzed against distilled H_2O for 12 hours and against 10 mM Tris-HCl buffer , pH 8.4 for 12 hours at 4°C. The dialyzed sample was loaded onto DEAE column at 4°C. The proteins were eluted from the column with the same buffer containing NaCl by linear and stepwise elution .

3.4.3. CM-Cellulose Chromatography

A. Materials: i) CM-Cellulose powder (20 gm)

ii) 0.2M HCl

iii) 0.2M NaOH solution

B. Procedure:

i) Activation of CM cellulose powder: The CM-cellulose powder was suspended in 0.2M HCl in a beaker and left it to swell for few hours. During swelling it was stirred gently at short intervals to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water several times until its pH reached near to neutrality. The gel suspension was then transferred to another beaker containing 0.2M NaOH solution and left for few hours. It was again washed with distilled water to neutralize its pH.

ii) Packing of the column: The activated CM-Cellulose suspension was taken in a filtering flask and deaerated by vacuum pump. A column of desired length was packed with the activated column material. Precaution was taken to avoid trapping of air bubbles during packing.

iii) Equilibration of the column: After packing, the column was equilibrated with 5mM sodium phosphate buffer, pH 6.5.

3.4.4. TEST OF PURITY

Sodium dodecyl sulfate polyacrylamide slab gel electrophoriesis (SDS-PAGE) Method

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

mass. Slab gel electrophoresis in presence of SDS therefore separated proteins almost exclusively on the basis of mass, with smaller polypeptides migrating more rapidly. Protein-SDS complexes will therefore all move towards the anode during electrophoresis and their movement is inversely proportional to the log 10 of their molecular weights. If standard proteins of known molecular weights. The protein pattern of the selected fractions was determined by 10% SDS-PAGE according to the method of Laemmli (1970).

3.5. CHARACTERIZATION OF PROTEIN:

3.5.1. MOLECULAR WEIGHT DETERMINATION:

3.5.1.1. By gel filtration method:

The molecular weight of the purified proteins were estimated from the data of gel filtration on Sephadex G-150 (0.75×100 cm) with Lysozyme (14600), trypsin inhibitor (20,000), egg albumin (45,000), bovine serum albumin (67,000) and β-galactosidase (116000) as reference proteins following the procedures as described by Andrews (1962).

3.5.1.2. By Sodium dodecyl sulfate polyacrylamide slab gel electroforesis method:

The molecular weights of the purified proteins were determined by this method that was described before for purity test of sample. {3.6.1 Sodium dodecyl sulfate polyacrylamide gel electroforesis (SDS-PAGE) method}

3.5.2. AFFINITY CHROMATOGRAPHY:

The pure protein obtained after dialysis against 5mM phosphate buffer saline, pH-7.2 was applied to Con A sepharose column previously equilibrated with the same buffer at 4°C. The absorbed protein was eluted from the column with the buffer containing 0.2M mannose. (Young, N.M. and Leon, M.A.; 1974).

3.5.3. HEMAGGLUTINATION STUDIES:

A. Materials :

(i) Phosphate buffer saline (PBS), pH-7.2.

(ii) 4% Rat red blood cells (RBC) in PBS

(iii) Protein solution.

B. Procedure:

Just before experiment, blood from albino rat was collected in centrifuged tube containing sufficient amount of pre-cooled 5 mM phosphate buffer saline pH 7.2. The blood sample was immediately centrifuged at $3X10^3$ g for 3 minutes. The supernatant was discarded and the cells were washed similarly for three times with the above buffer. Finally a 4% suspension (W/V) of RBC was prepared and the hemagglutination was performed in siliconized test tubes (0.5 x 4 cm) as follows:

0.2 ml of 4 % RBS were mixed with 0.2 ml of protein solution in PBS and mixed well by gentle stirring. The mixture was incubated at 30°C for an hour. A control containing 0.2 ml of PBS, pH 7.2, instead of protein solution and 0.2 ml cell suspension were used as reference. After 1 hour incubation, the sedimented erythrocytes were gently mixed with the supernatant and one drop of this suspension was examined under microscope. Results were recorded as 3^+, 2^+, 1^+ (Read, W. P., (1981).

The agglutinating activity was expressed as the titre, the reciprocal of the greatest dilution at which visible agglutination could be detected. The specific activity was expressed as titre / mg of protein.

3.5.4. HEMAGGLUTINATION INHIBITION STUDIES:

A. Materials:

(i) 5 mM phosphate buffer saline, pH-7.2.

(ii) Different sugar solutions.

B. Procedure:

The hemagglutination-inhibition test was performed in the presence of different sugars as described below. Protein solutions (0.1 ml) containing minimum concentration of protein needed for visible agglutination were added to 0.1 ml of sugar solutions of various concentrations and mixed gently, and then 0.2 ml of 4% RBC in PBS was mixed and incubated at 30°C for an hour. Reactions were compared with a positive control (0.1 ml protein + 0.1 ml buffer + 0.2 ml 4% RBC) and a negative control (0.2 ml PBS + 0.2 ml 4% RBC) as reported by Alkinson *et al.* (1980).

3.5.5. TEST FOR GLYCOPROTEIN AND ESTIMATION OF SUGAR:

3.5.4.1. Phenol - Sulfuric acid:

Phenol in the presence of sulfuric acid can be used for quantitative colorimetric microdetermination of sugars and their methyl derivatives, oligosaccharide and polysaccharides as described by Dubois *et al.* (1956). This method was also employed for detecting the presence of sugar in protein.

A. Materials:

(i) 5% phenol (in water)

(ii) Conc. sulfuric acid

(iii) Protein solution

B. Procedure :

The protein solution (0.1 ml from protein solution of 0 .075 - 0.097mg / ml) was taken in a test tube and made upto 2 ml by distilled water. Then 1 ml of 5% phenol was added to it and finally 5 ml of conc. H_2SO_4 was added rapidly .To obtain good mixing the stream of acid being directed against the liquid surface rather than against the side of the test tube . The tube was allowed to stand for 10 minutes. Then it was shaken and kept in the dark at 25 to 30°C for 20 min. It was taken out and the absorbance of the solution was measured at 490 nm. The conc. of sugar was then estimated from a standard curve which was constructed by using glucose as standard sugar.

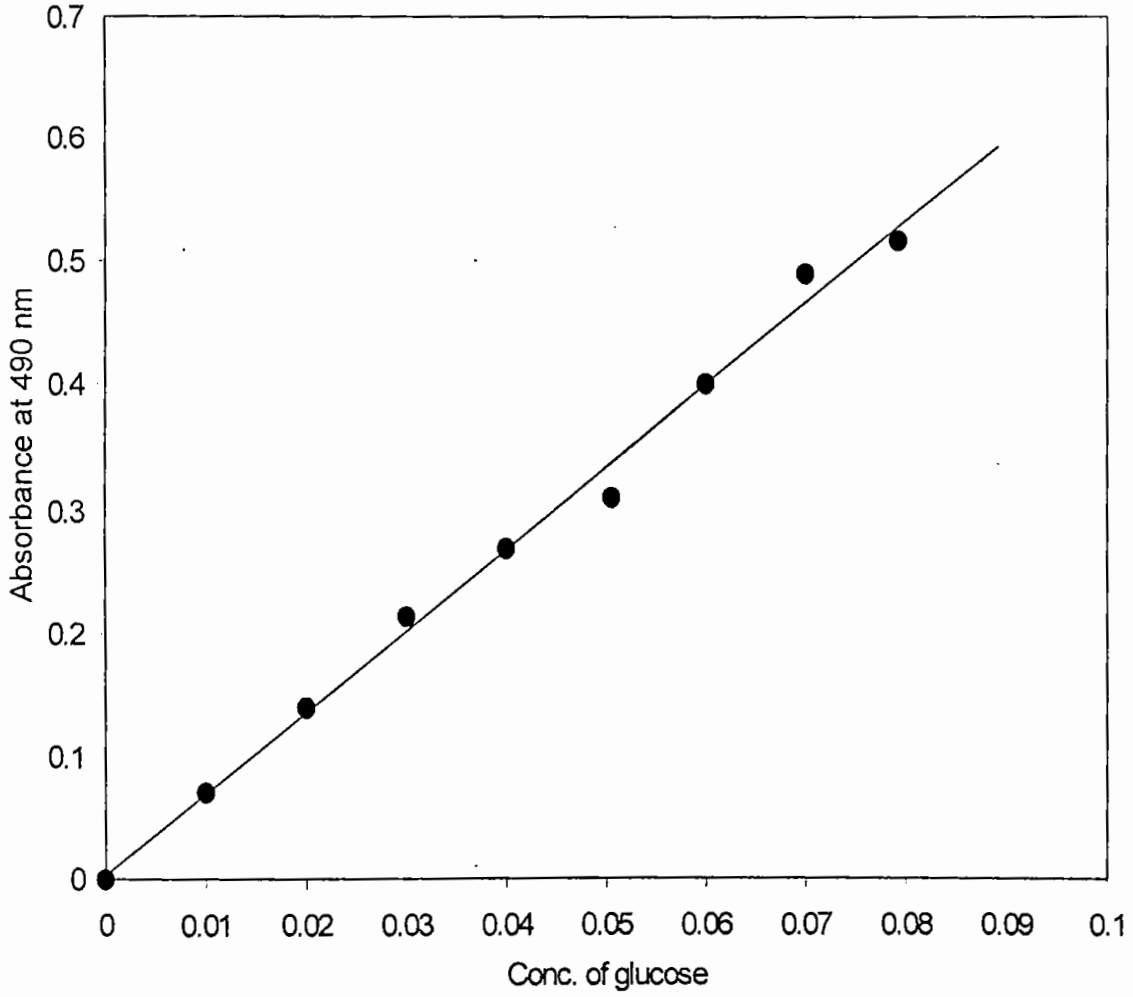


Fig. 3.1. Standard curve for estimation of sugar present in glycoprotein.

3.5.6. TEST FOR GLYCOPROTEIN BY PERIODIC ACID-SCHIFF'S (PAS) METHOD:

Khomei Yanagi *et al.,* 1990 described a sensitive PAS staining method for detection of sugar in glycoprotein.

A. Reagents

i) Fuchsin-sulphite (Schiffs reagent)

ii) 2N HCl

iii) 12.5% Trichloro acetic acid (TCA)

iv) 0.5% sodium metabisulphite

v) 5% acetic acid

vi) Potassium metabisulphite

B. Preparation of Schiff's Reagent (staining solution)

2 gm of basic fuchsin was dissolved in 400 ml of distilled H_2O with warming, then cooled and filtered. 10 ml of 2N HCL and 4 gm potassium metabisulphite were added to it and was placed in a stoppered bottle and kept at cool dark place for overnight. 1 gm activated charcoal was added to it and then filtered. After filtering sufficient amount of 2N HCl (10 ml or more) was added to it until a drop dried on a glass slide does not turn red. Stored the reagent in a stoppered colored bottle and kept at cool dark place. The reagent might be discarded if it turned pink.

C. Staining method:

After disc gel electrophoresis, the gels were stained in the following successive methods.

i) The gels were immersed in 12.5% TCA for 30 minutes.

ii) The gels were then rinsed in distilled H_2O for 25 to 30 seconds.

iii) The gels were immersed in 1% periodic acid in 3% aqueous acetic acid for 50 minutes.

iv) The gels were washed thoroughly with distilled H_2O for overnight.

v) The gels were then transferred into fuchsin sulphite stain in dark and kept for 50 minutes.

vi) The gels were then washed with three changes (10 min. each) of 0.5% sodium metabisulphite solution.

vii) The gels were washed in frequent changes of distilled H_2O until excess stain was removed.

viii) Finally, gels were stored in 5% acetic acid.

3.5.7. DETERMINATION OF PROTEIN CONCENTRATION BY THE FOLIN-LOWRY METHOD (Lowry *et al.* 1951):

A. Materials:

(i) Alkaline sodium carbonate solution (20 gm / litre Na_2CO_3 in 0.1 mol/litre NaOH)

(ii) Copper sulphate and Sodium potassium tartrate solution (5 gm/litre $CuSO_4.5H_2O$ in 10 gm/litre Na-K tartarate). Freshly prepared,

(iii) Alkaline solution: Mixture of solution 1 & 2 in the proportion of 50:1, respectively.

(iv) Folin-Ciocalteau's reagent (Diluted with equal volume of H_2O, just before use).

(v) Standard protein (bovine serum albumin 1 mg / ml in dist. H_2O) solution.

B. Method:

From standard protein solution 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 ml were taken in different test tubes and made the volume up to 1 ml by distilled water. Then 5 ml of the alkaline solution (solution-3) was added to the protein solution in different test tubes and mixed thoroughly. It was allowed to stand at room temperature for 10 minutes. Then 0.5 ml of diluted Folin-Ciocalteau's reagent was added rapidly with immediate mixing and left for 30 minutes. The dark blue color formed was measured at 650 nm against the appropriate blank. By applying the same procedure described above the absorbance of purified protein solution was measured and the concentration of protein was determined by constructing a standard curve.

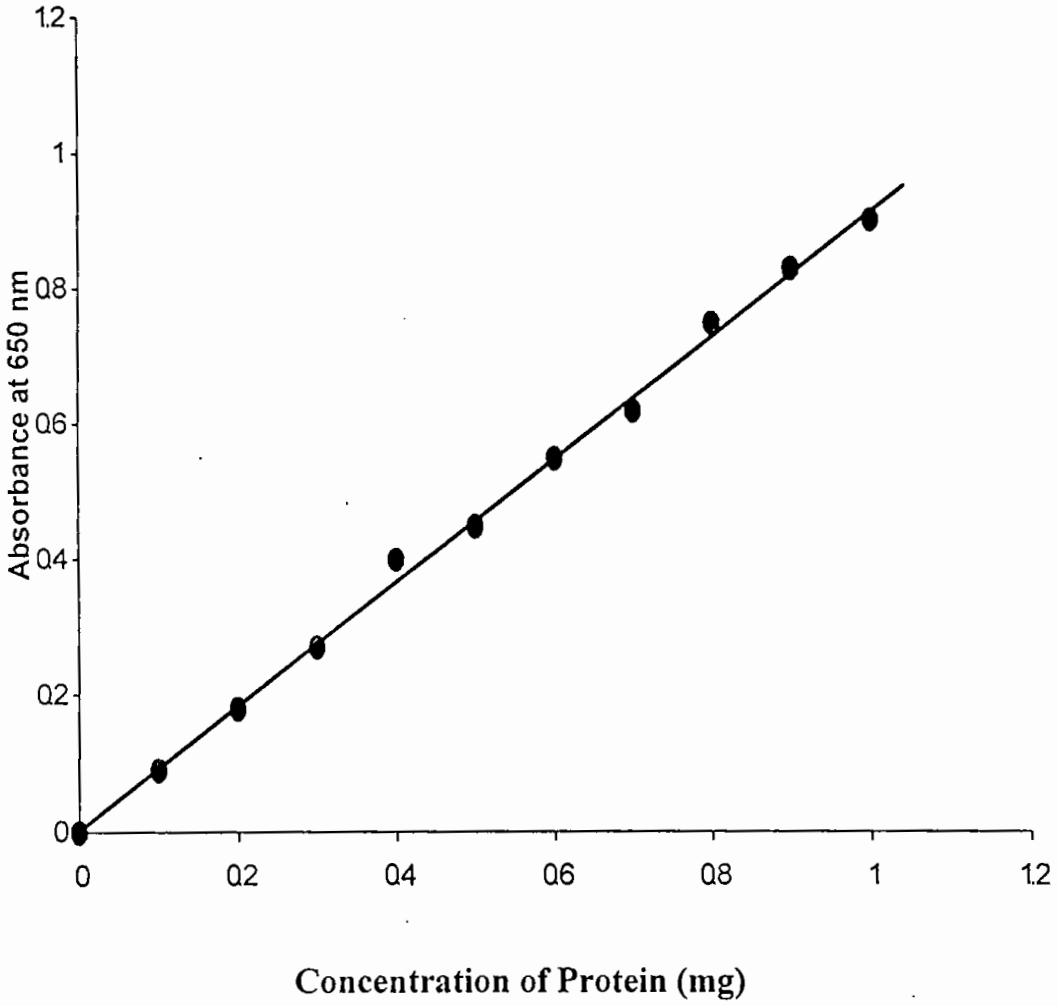


Fig. 3.2. Standard curve for estimation of the concentration of proteins.

3.5.8. BRINE SHRIMP LETHALITY: A RAPID GENERAL BIOASSAY FOR CYTOTOXIC EFFECT:

Brine shrimp lethality bioassay is a recent development by Mayer *et al.,* (1982) in the bioassay for bioactive compounds, which indicates cytotoxicity as well as a wide range of pharmacological activities e. g anticancer, antiviral etc. Bioactive compounds are almost always toxic in high doses. Here in vivo, lethality of simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening and fractionation in the discovery of new bioactive products.

There is a positive correlation between brine shrimp toxicity and cytotoxicity. This bioassay is indicative of cytotoxicity and a wide range of pharmacological activities of natural products.

Materials:

1) *Artemia salina* Leach (brine shrimp eggs)

2) NaCl

3) Pipettes (5 ml & 1 ml)

4) Micro pipette (10-100 µl adjustable)

5) Two drum vials

6) Magnifying glass

7) Small tank with perforated dividing dam to grow shrimp, including cover and lamp to attract shrimp.

Procedures:

A) Preparation of sea water: 38 gms of NaCl was weighed, dissolved in one liter of distilled water and then filtered off.

B) Hatching of brine shrimp eggs: Sea water was taken in small tank and shrimp eggs were added to one side of the divided tank which was covered. The shrimps were allowed for two days to hatch and matured as nauplii. The hatched shrimps were attracted to the lamp on the other side of the divided through the perforations in the dam. These nauplii were taken for bioassay.

C) Preparation of sample solution: 2ml of each protein in Tris-HCl buffer (protein conc. 1 mg/ml) were dialyzed separately against distilled water for 3 hours at 4°C.

D) Application of test solution and nauplii in the vials: At room temperature 2, 5, 10, 20, 30 & 40 µl of the stock solutions were taken in vials and 5 ml of the sea water was added to each vial containing 10 brine shrimp nauplii. So, the concentrations of sample in the vials were 0.4, 1, 2, 4, 6 and 8 µg/ml respectively. Three vials were used for each concentration and control was used containing 10 nauplii in 5 ml of sea water.

E) Counting of nauplii: After 24 Hours incubation the vials were observed and the number of survivors in each vial were counted using magnifying glass and noted. From this data, the percentage of mortality of the nauplii was calculated at each concentration.

3.5.9. IDENTIFICATION OF REDUCING SUGAR PRESENT IN THE PURIFIED PROTEIN BY THIN LAYER CHROMATOGRAPHY (TLC):

Ascending one dimensional thin layer chromatography was performed in an attempt to identify the sugar components present in the hydrolyzed protein solution.

A. Materials:

i) Standard sugars:

D-Glucose, D-galactose, D-arabinose, D-mannose and D-ribose (0.01 mg/ml) were used as standard sugars for the experiment.

ii) Preparation of protein sample: The protein sample (1 ml) of having concentration (A_{280} = 0.4- 0.5) was evaporated to dryness by vacuum pump. Then 2 ml of 4N HCl was added to it and after deaeration the tube was sealed by heating. It was then heated at 100^0C for four hours. The hydrolyzate was evaporated to dryness and dissolved in a few drops of distilled water and used for analysis.

iii) Stationary phase: Activated silica Gel-G.

iv) Mobile phase: Isopropanol : Acetic acid : Water (3:1:1)

v) Spray reagent : (Aniline-Phthalate) (Partridge 1949; Parchke 1965)

Phthalic acid	4.0 gm
Aniline	2.5 ml
Water saturated butanol	250 ml

B) Procedure:

i) Preparation of plates: The plates were prepared and activated by applying conventional procedure.

Plate size:	(20x20) cm
Thickness:	0.5 mm
Activation:	110^0C for 1 hour

ii) Chamber saturation: Glass chamber with airtight glass lid was used for development of the plates. The chamber was saturated with the vapors of the selected solvent system in the usual manner.

iii) Resolution of compounds: A small spot of the standard sugar solution was applied at 2 cm above the lower edge of the activated silica gel-G plate and about 4 cm apart by means of a capillary tube. The spot diameter was kept below 0.5 cm. A spot of the hydrolyzed protein solution was also applied on the side of the plate as for standard. A straight was cut 2 cm below the upper edge of the plate and it was the indication for the solvent front. Then the spotted plate was placed gently in the chromatographic tank containing the selected solvent system. When the developing mobile phase reached the marked point, the plate was taken out and dried in the air.

iv) Detection of compounds: Aniline -phthalic acid mixture was sprayed on the plate. The plate was heated at 100^0C for 10 minutes and locations of spots were marked.

v) Calculation of R_f values: The R_f values were determined by using the following formula:

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

RESULTS

3.6. PURIFICATION OF *Potamogeton nodosus* ROOTSTOCK PROTEINS:

3.6.1. GEL FILTRATION:

The 100% ammonium sulfate saturated crude protein extract after dialysis against distilled water and against 10 mM Tris-HCl buffer, pH 8.0 was applied to a Sephadex G-50 column at 4°C which was previously equilibrated with the same buffer. As shown in Fig.3.3, the components of the crude protein extract were eluted as three fractions, F-1, F-2 and F-3. All the three fractions, as they contained hemagglutination property, were used for experimental purposes. The areas as indicated by solid line of different fractions were pooled, precipitated by ammonium sulfate (100 % saturation) and subjected to further purification by ion-exchange chromatography.

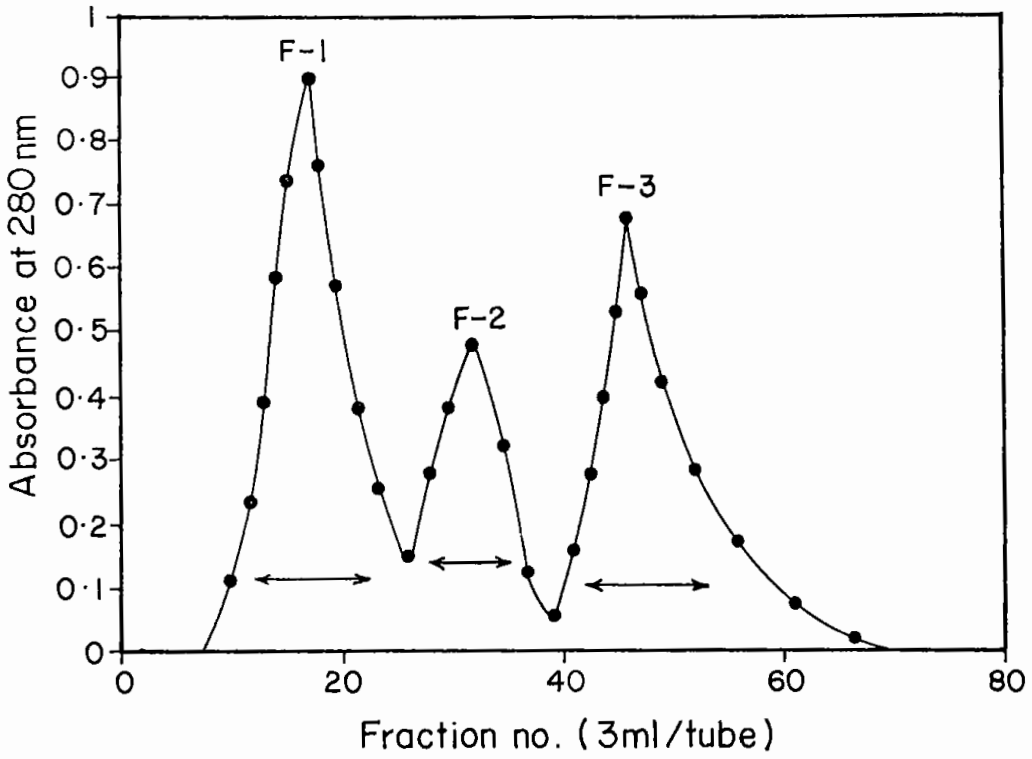


Fig. 3.3. Gel filtration of 100% $(NH_4)_2SO_4$ saturated crude protein extract on Sephadex G-50. The crude extract (82 mg) was applied to the column (2.5 X 100 cm) pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.0, at 4°C and developed with the same buffer.

3.6.2. DEAE-CELLULOSE CHROMATOGRAPHY OF F-1, F-2 AND F-3 FRACTIONS:

The ammonium sulfate precipitate of F-1, F-2 and F-3 fractions as obtained by centrifugation at 8000g were dissolved separately in minimum volume of distilled water, dialyzed against distilled water for 12 hours and against 10 mM Tris-HCl buffer, pH 8.4 at 4°C for 12 hours with three changes of the buffer. After centrifugation, the clear supernatants were applied separately to a DEAE-Cellulose column at 4°C previously equilibrated with 10 mM Tris-HCl buffer, pH 8.4 and eluted by a linear gradient of NaCl from 0 to 0.8 M, 0 to 0.4 M, 0 to 0.3 M respectively in the same buffer. As shown in Fig. 3.4, Fig. 3.5 and Fig. 3.6, all the three fractions were eluted from the column by almost single broad peak, indicating that the fractions might be contained more than one components. In order to separate these components, the elution was carried out stepwise with increasing concentrations of NaCl in the same buffer.

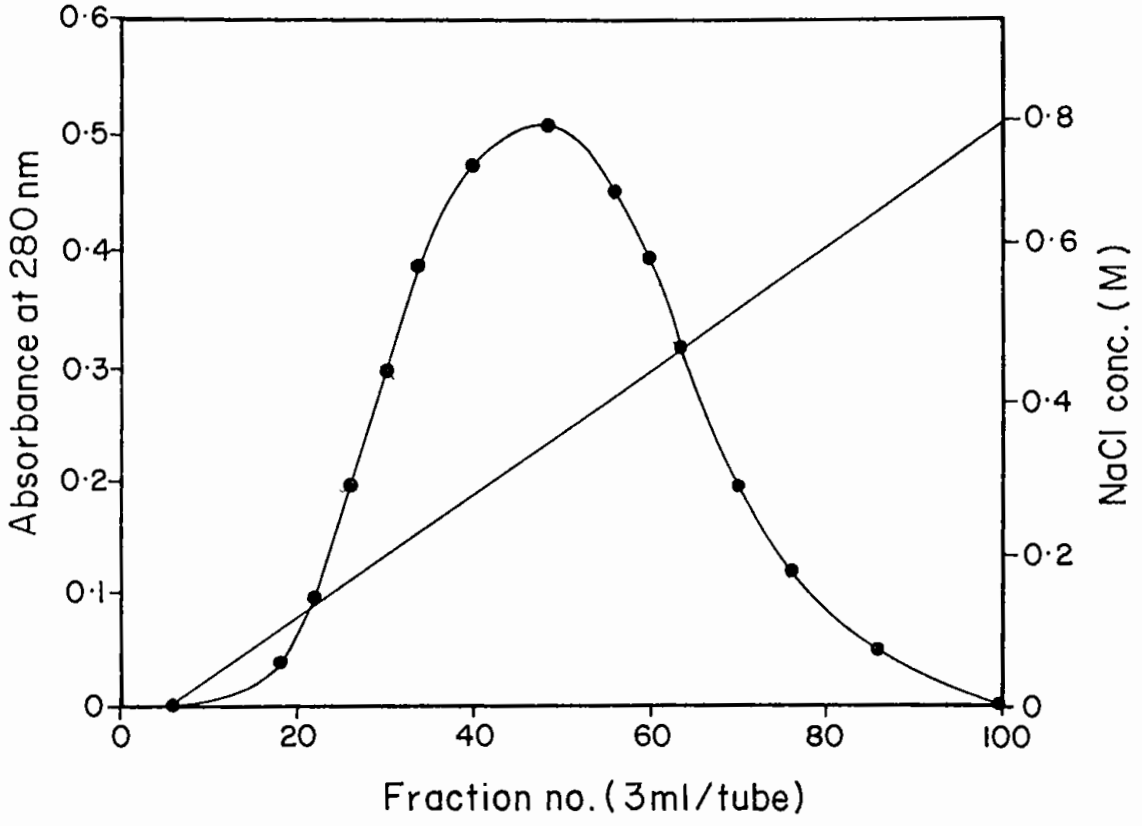


Fig. 3.4. Ion exchange chromatography of F-1 fraction on DEAE-cellulose. F-1 fraction (20mg) obtained by gel filtration, was applied to the column (2.1 X 24 cm) prewashed with 10mM Tris-HCl buffer, pH. 8.4 at 4°C and eluted by a linear gradient of NaCl in the same buffer.

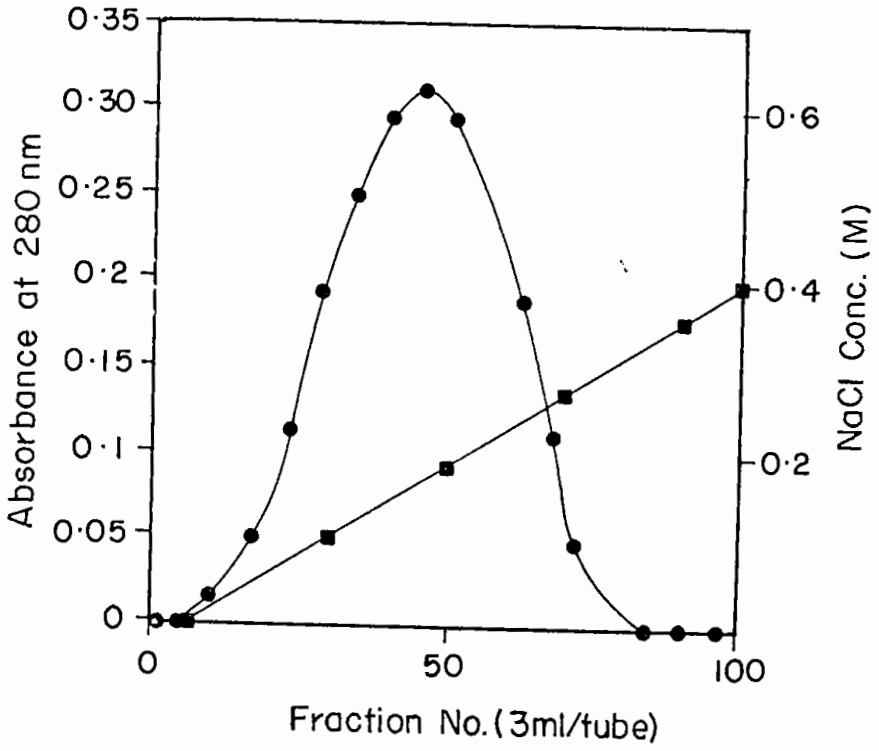


Fig. 3.5. Ion exchange chromatography of F-2 fraction on DEAE-cellulose. F-2 fraction (22mg) obtained by gel filtration, was applied to the column (2.1 X 24 cm) prewashed with 10mM Tris-HCl buffer, pH. 8.4 at 4°C and eluted by a linear gradient of NaCl in the same buffer.

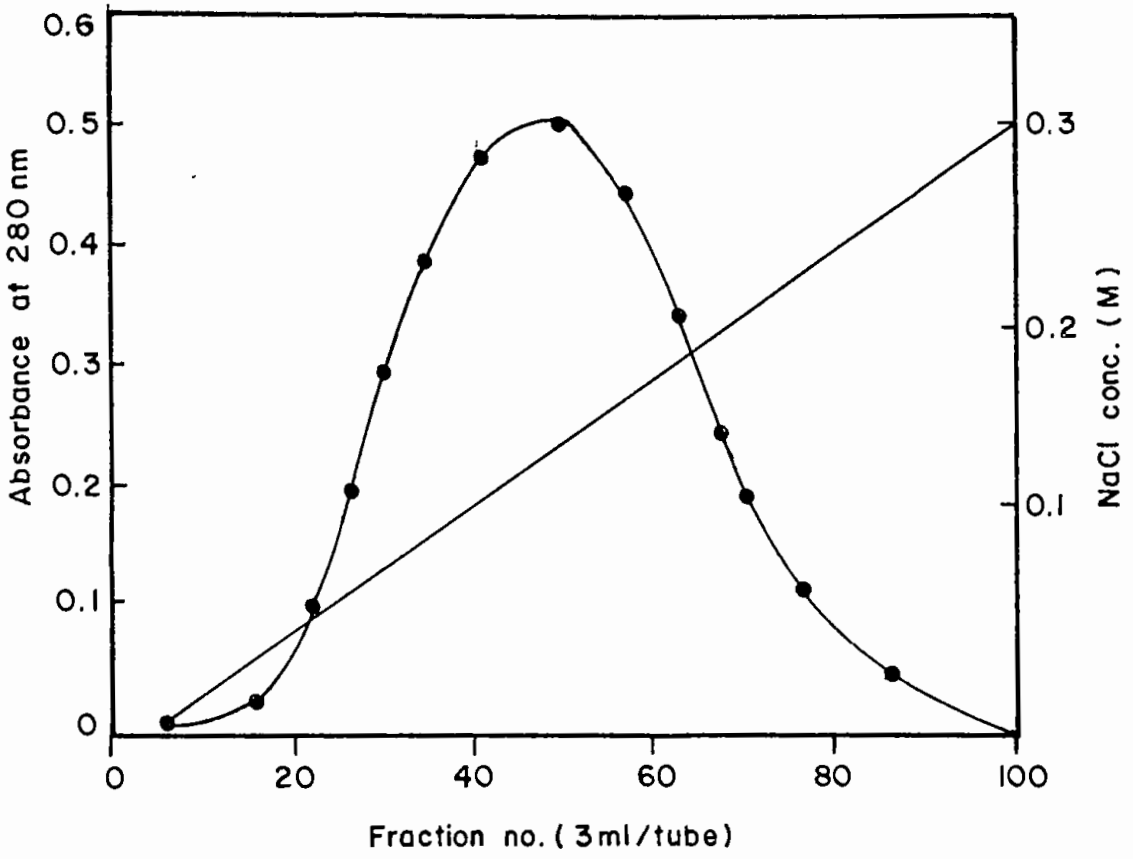


Fig. 3.6. Ion exchange chromatography of F-3 fraction on DEAE-cellulose. F-3 fraction (22mg) obtained by gel filtration, was applied to the column (2.1 X 24 cm) prewashed with 10mM Tris-HCl buffer, pH. 8.4 at 4°C and eluted by a linear gradient of NaCl in the same buffer.

As shown in Fig.3.7, most of the components of fraction, F-1 were bound to the DEAE-cellulose column while a small unbound fraction i.e. F-1a was eluted from the column by the buffer only. On the other hand, the major fractions, F-1b, F-1c and F-1d were eluted from the column by same buffer containing 0.05 M, 0.1 M and 0.4 M NaCl respectively while the minor fraction, F-1e was eluted from the column by the buffer containing 0.8 M NaCl. Further it was found that the fractions F-1b, F-1c and F-1d possessed biological activities, so the purity of these fractions were detected by SDS-PAGE, while F-1a and F-1e fractions were discarded as they contained no biological activities.

DEAE-chromatography of F-1 and F-3 fractions

As shown in Fig. 3.8, the second fraction i.e. F-2 obtained after gel filtration was bound completely to DEAE-cellulose column and the bound components were separated into one main fraction, F-2a and one minor fraction, F-2b which was eluted from the column by the same buffer containing 0.2 M and 0.4 M NaCl respectively. Of these two fractions, only F-2a showed hemagglutinating activity and its purity was checked by SDS-PAGE. Like F-2, F-3 fraction was also bound completely to the DEAE-cellulose and the components were separated into one major fraction, F-3a and two minor fractions, F-3b and F-3c which were eluted from the column by 10mM Tris-HCl buffer, pH 8.4 containing 0.06, 0.15 and 0.25M NaCl respectively (Fig. 3.9). The fraction, F-3a and F-3b possessed hemagglutinating activities and their purity were checked by SDS-PAGE.

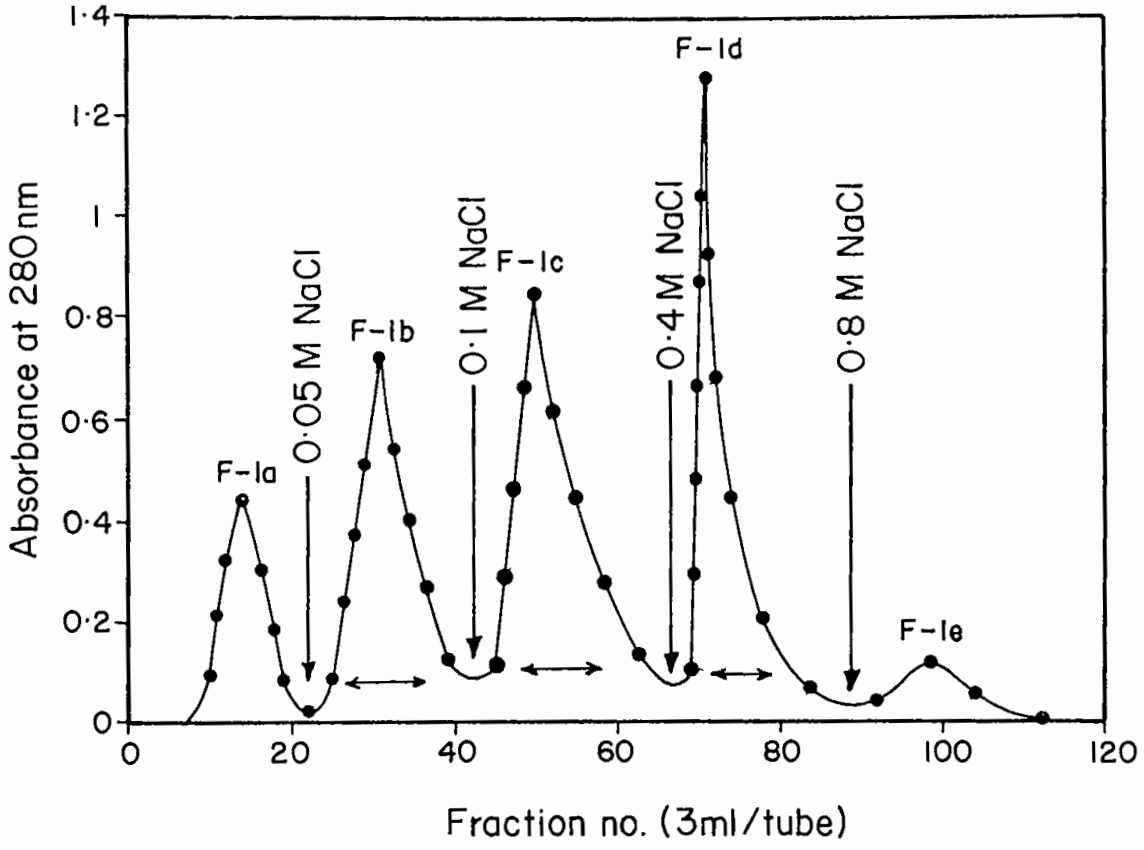


Fig. 3.7. Ion exchange chromatography of F-1 fraction on DEAE-cellulose.F-1 fraction (52 mg) obtained by gel filtration , was applied to the column (2.1 X 24 cm) prewashed with 10mM Tris-HCl buffer, pH. 8.4 at 4°C and eluted by stepwise increases of NaCl concentrations in the same buffer.

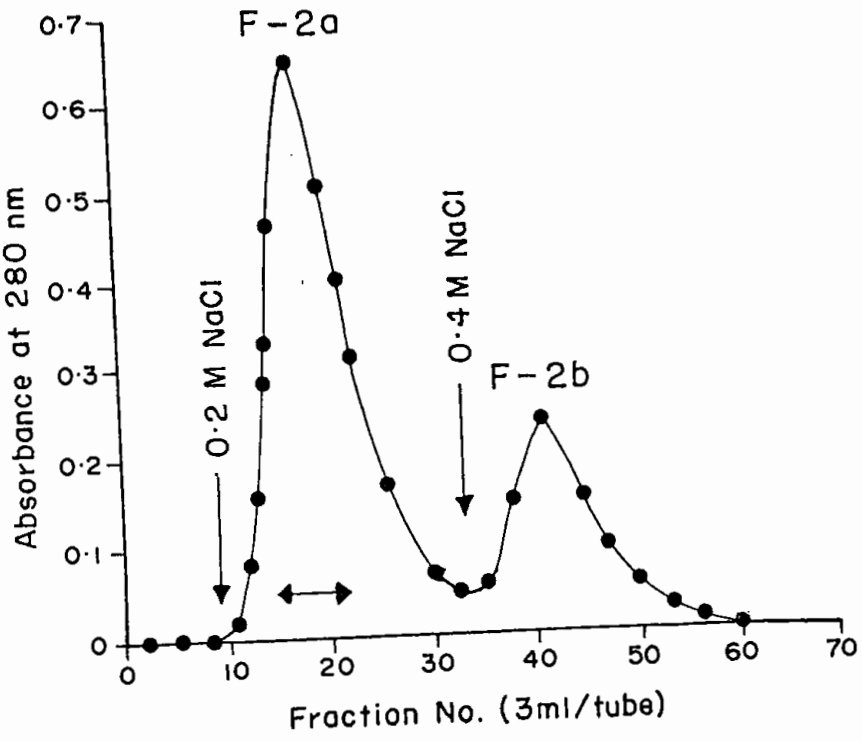


Fig. 3.8. Ion exchange chromatography of F-2 fraction on DEAE-cellulose.F-2 fraction obtained (20mg) by gel filtration, was applied to the column (2.1 X 24 cm) prewashed with 10mM Tris-HCl buffer, pH. 8.4 at 4°C and eluted by stepwise increases of NaCl concentrations in the same buffer.

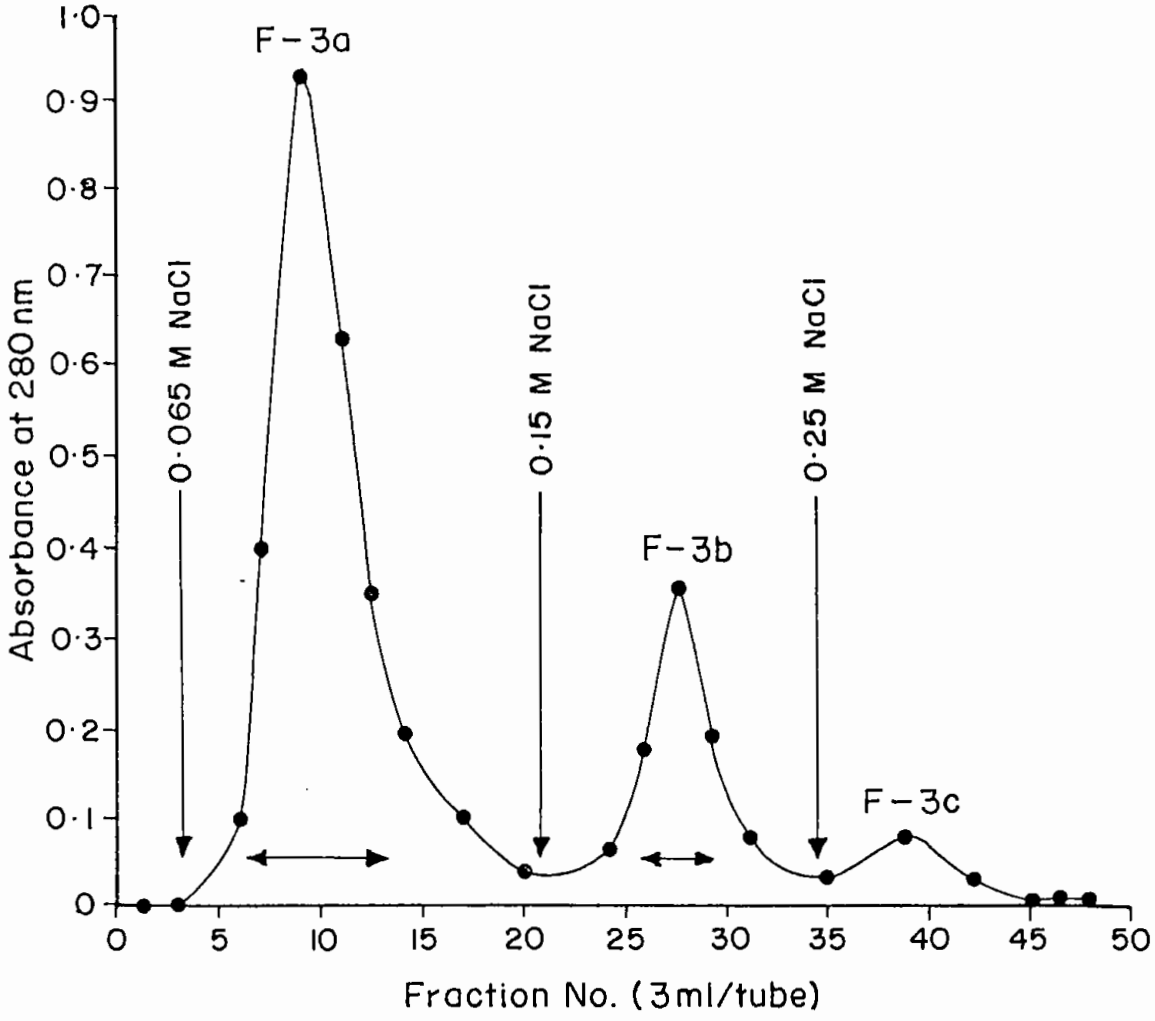


Fig. 3.9. Ion exchange chromatography of F-3 fraction on DEAE-cellulose.F-3. Fraction F-3 (30mg) obtained by gel filtration , was applied to the column (2.1 X 24 cm) prewashed with 10mM Tris-HCl buffer, pH. 8.4 at 4°C and eluted by stepwise increases of NaCl concentrations in the same buffer.

3.6.3. SDS-PAGE:

SDS polyacrylamide slab gel electrophoresis of the different protein fractions obtained from the DEAE cellulose chromatography i.e. , F-1b, F-1c, F-1d, F-2a, F-3a and F-3b were performed on 10% gel at pH 8.4 and the photographic representation of the electrophoretic patterns are shown in Fig. 3.10. From the results it might be concluded that the fractions, F-1b, F-1d, F-3a and F-3b contained pure protein as they gave single band on the gel while the fraction F-1c and F-2a are not pure as they gave more than one band on the gel (Fig. 3.10).

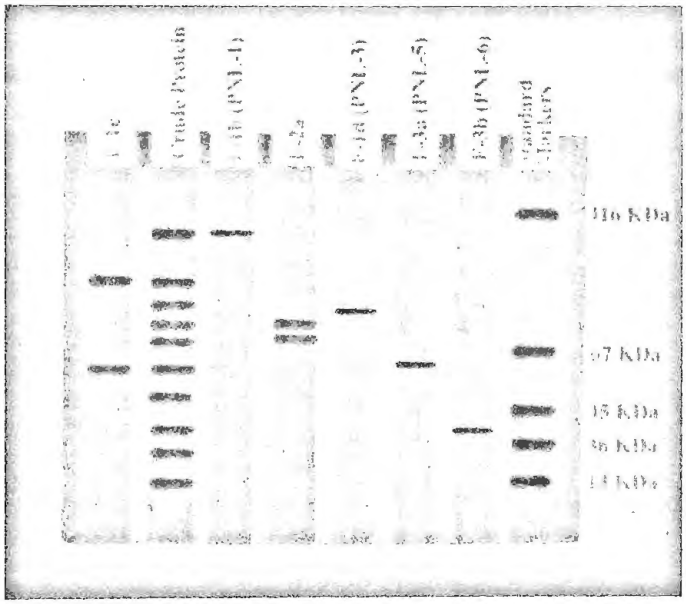


Fig. 3.10. SDS-Polyacrylamide slab gel electrophoretic patterns of different protein fractions at room temperature

Buffer = 10 mM Tris HCl buffer, pH 8.4

Concentration of protein = 75 μg

3.6.4. CM-CELLULOSE CHROMATOGRAPHY OF F-1C AND F-2A FRACTIONS:

The fraction of F-1c and F-2a as indicated by solid bar were pooled separately and dialyzed against distilled water for 12 hours and against the eluting buffer (5mM sodium phosphate buffer, pH 6.5) for 12 hours at 4^0C. After centrifugation the clear supernatant was applied to the CM-cellulose column separately. The columns were previously equilibrated with the above mentioned buffer and eluted with the same buffer at 4^0C.

As shown in the Fig. 3.11, the bulk of F-1c fraction was not bound to the CM-cellulose column and eluted from the column by the buffer only as a single peak (F-1c′) while a minor fraction i.e. F-1c″ was eluted from the column as broad peak by the buffer containing 1.0 M NaCl. It was found that the fraction which was eluted by the buffer only contained hemagglutinating activity and its purity was checked by SDS polyacrylamide slab gel electrophoresis.

Like F-1c, F-2a fraction was also separated into two fractions F-2a′ and F-2a″ (Fig.3.12). Of these fractions, F-2a′ was eluted by the buffer only while F-2a″ was eluted by the buffer containing 0.35M NaCl. Only F-2a′ contained hemagglutinating activity and its purity was also checked by SDS polyacrylamide slab gel electrophoresis.

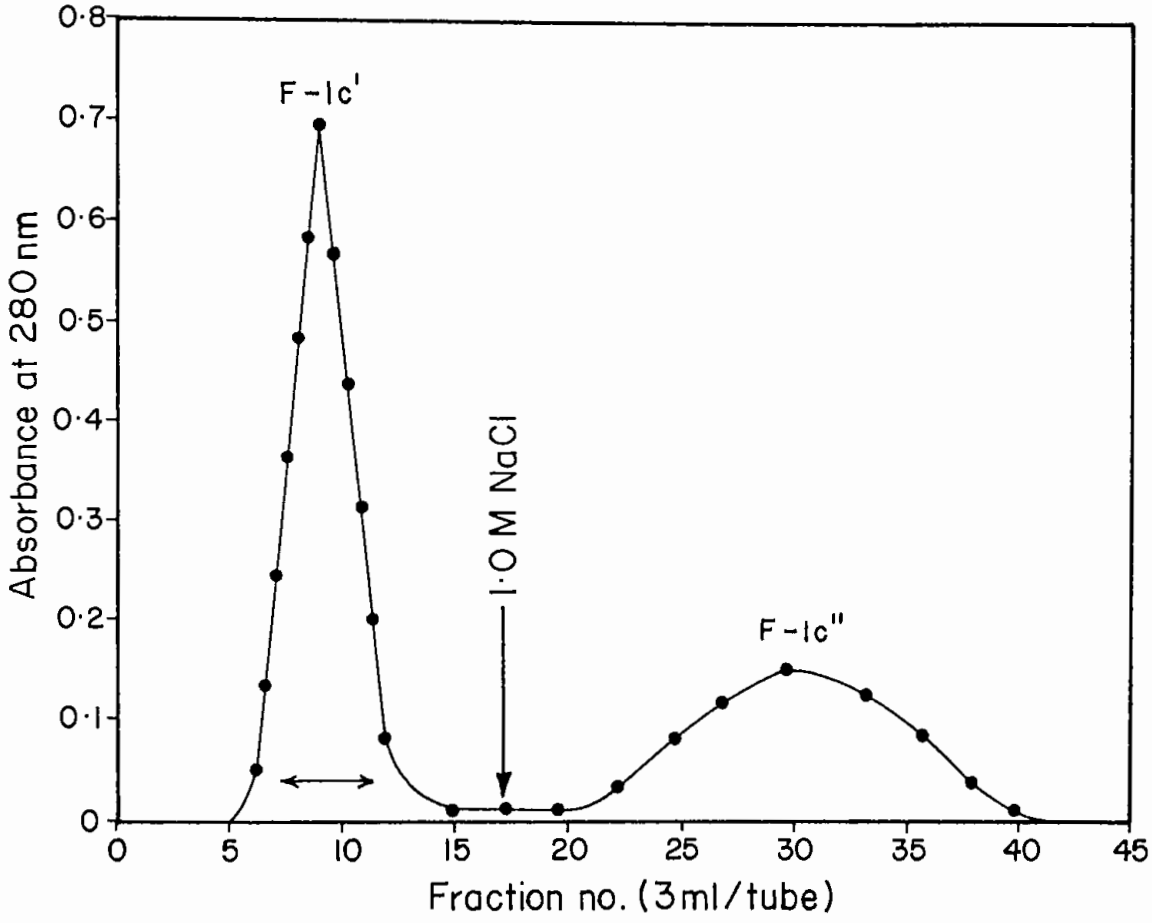


Fig. 3.11. Ion exchange chromatography of F-1c fraction on CM-cellulose. F-1c (10.4mg) fraction obtained by DEAE-cellulose chromatography, was applied to the column (2.1X 24cm), prewashed with 5mM phosphate buffer, pH 6.5 at 4^0C and then eluted by 1.0 M NaCl in the same buffer.

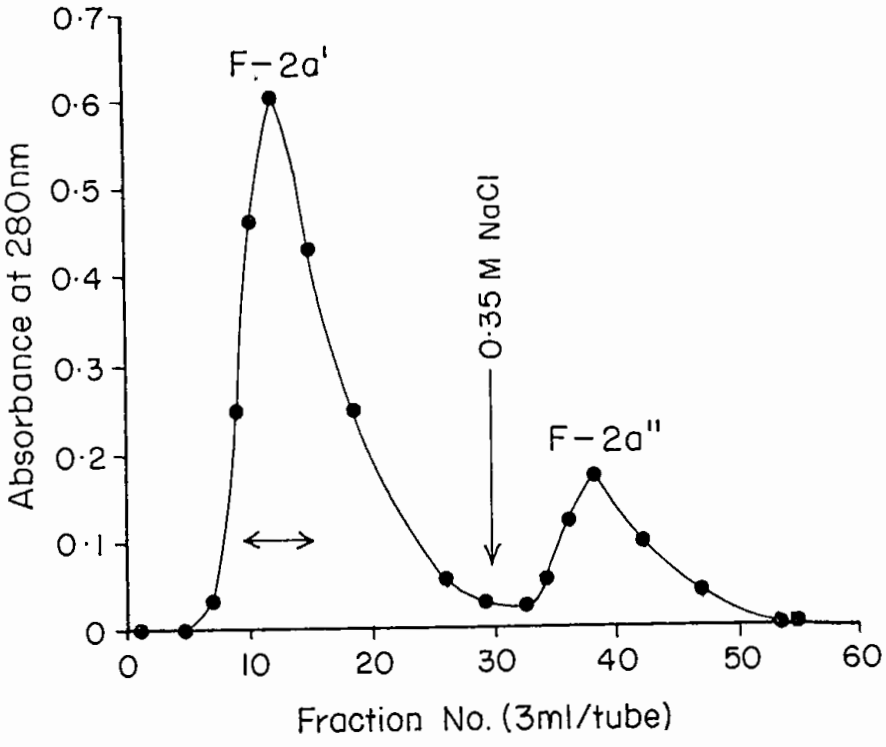


Fig. 3.12. Ion exchange chromatography of F-2a fraction on CM-cellulose. F-2a (10mg) fraction obtained by DEAE-cellulose chromatography, was applied to the column (2.1 × 24cm) prewashed with 5mM phosphate buffer, pH 6.5 at 4^0C and then eluted by 0.35M NaCl in the same buffer.

3.6.5. CHECK OF PURITY:

The fraction F-1c′ and F-2a′ must be contained pure protein as they gave single band on 10% SDS-PAGE (Fig. 3.13).

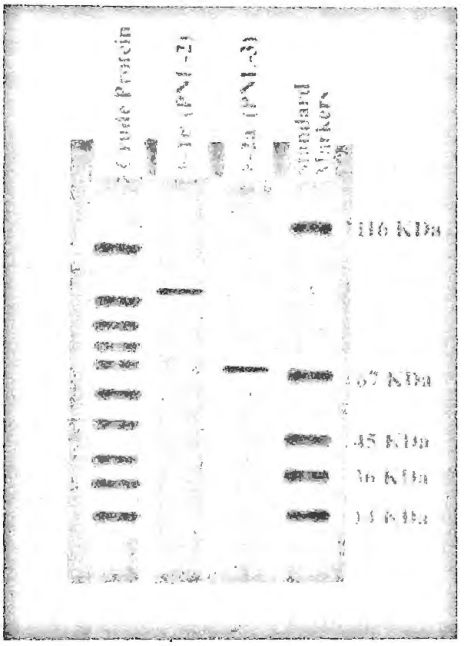


Fig. 3.13. SDS-Polyacrylamide slab gel electrophoretic patterns of fraction, F-1c′ and F-2a′ at room temperature.

Buffer = 10 mM Tris HCl buffer, pH 8.4

Concentration of protein = 75 μg

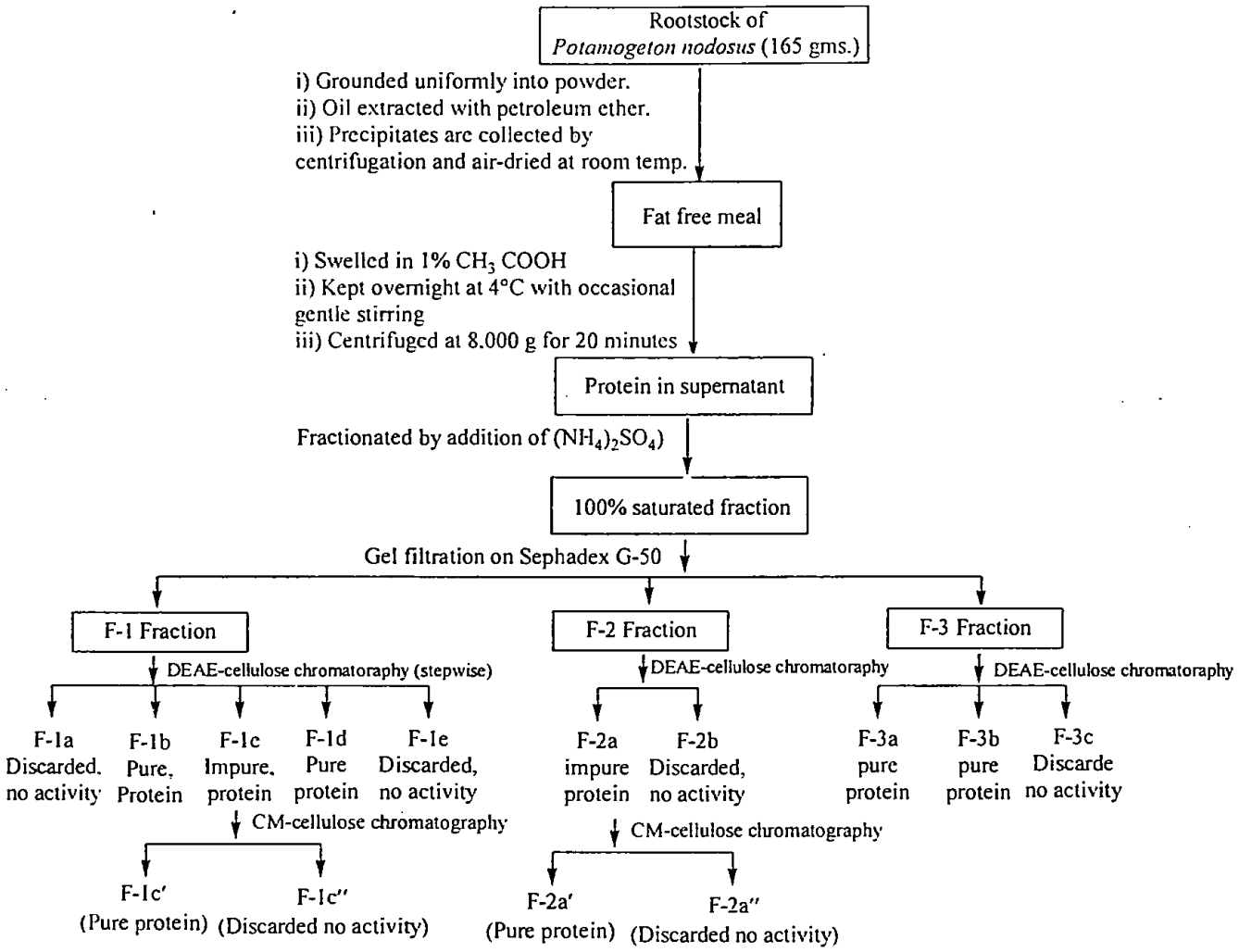


Fig: 3.14. Schematic representation of the protein purification steps.

The overall purification data of *Potamogeton nodosus* roototock proteins were summariged in Table-3.2. 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-1C′ showed maximum hemagglutination activity with a purification fold of 16.06 while the fraction F-1b, F-1d, F-3a, F-3b, and F-2a′ showed the purification fold of.16.61, 9.56, 11.6, 14.36, 16.06 and 15.00 respectively. Althoug the yield of these proteins were decreased after each subsequent purification steps and more than 97% 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 3.2. Purification of *Potamogeton nodosus* rootstock proteins.

Fraction		Total protein (mg)	Total Hemagglutination activity (titre)	Specific activity (titre / mg)	Yield (%)	Purification (fold)
Crude extract		980	4900	5.0	100	1.00
100% $(NH_4)_2 SO_4$ saturated		230	2070	9.0	42.24	1.8
After gel filtration on Sephadex G-50	F-1	31.2	510	16.34	10.40	3.268
	F-2	24.1	420	17.42	10.08	3.48
	F-3	26.4	421	15.95	8.59	3.19
DEAE cellulose fraction	F-1b	3.01	250	83.05	5.10	16.61
	F-1c	6.2	200	32.25	4.08	6.45
	F-1d	2.3	110	47.83	2.24	9.56
	F-2a	6.0	288	48.0	5.87	9.6
	F-3a	2.5	147	58.8	30.00	11.6
	F-3b	2.2	158	71.82	3.22	14.36
CM cellulose fraction	F-1c′	2	160	80.33	3.26	16.06
	F-2a′	2	280	75.00	5.71	15.00

Hemagglutination activity (titre) = Reciprocal of highest dilution showing visible Hemagglutination .

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

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

3.7. CHARACTERIZATION OF PURIFIED *Potamogeton nodosus* ROOTSTOCKS PROTEINS:

3.7.1. MOLECULAR WEIGHT DETERMINATION:

I) By gel filtration method:

The molecular weight of the proteins were determined by gel filtration on Sephadex G-150. The standard curve was constructed by plotting the log of molecular weight of the proteins against elution volume using Lysozyme , Trypsin inhibitor , Egg-albumin , Bovine serum albumin and β -Galactosidase as reference proteins (Fig. 3.15). The calculated molecular weight of F-1b, F-1c′, F-1d, F-2a′, F-3a and F-3b were found to be 98000, 91000, 77500, 68000, 62500 and 38500 respectively (Fig. 3.15).

II) By SDS polyacrylamide slab gel electrophoresis :

Molecular weight of the purified proteins were determined by SDS-Polyacrylamide Slab gel electrophoresis at pH 7.2 and β-galactosidase, Lysozyme, BSA and egg albumin were used as reference proteins. The molecular weight of the proteins were calculated from the standard curve of reference proteins which was constructed by plotting the log of molecular weight against relative motilities of the proteins on the gel after electrophoresis (Fig. 3.16). The molecular weight of F-1b, F-Ic′, F-id, F-2a′, F-3a and F-3b were estimated to be 97,500, 83,000; 77,000; 67,500; 62,000 and 38100 respectively (Fig. 3.17). The subunit structure of other proteins were also determined by SDS-Polyacrylamide slab gel electrophoresis in the presence of 0.1% SDS and 1% β-mercaptoethanol. F-Ib protein was dissociated from each other, gave four distinct bands (Fig. 3.18), corresponding to molecular weight of 30,200; 25,000; 22,000 and 18,000 indicating that the F-Ib are tetramer in nature and subunits are held together by non-ionic hydrophobic

interactions. F-1c′, gave single band (Fig. 3.18) corresponding to molecular wt of 22,000 indicating that this protein contains four subunits of equal size which are held together by S-S bond. Again F-1d and F-2a′ gave two bands (Fig. 3.18) corresponding to molecular weight of 41,600; 35,000 and 35,300; 31,800 respectively. F-3a, and F-3b proteins contained only one subunit as they gave single band on SDS-PAGE corresponding to molecular weight of 62,000 and 38,100 (Fig. 3.18) respectively.

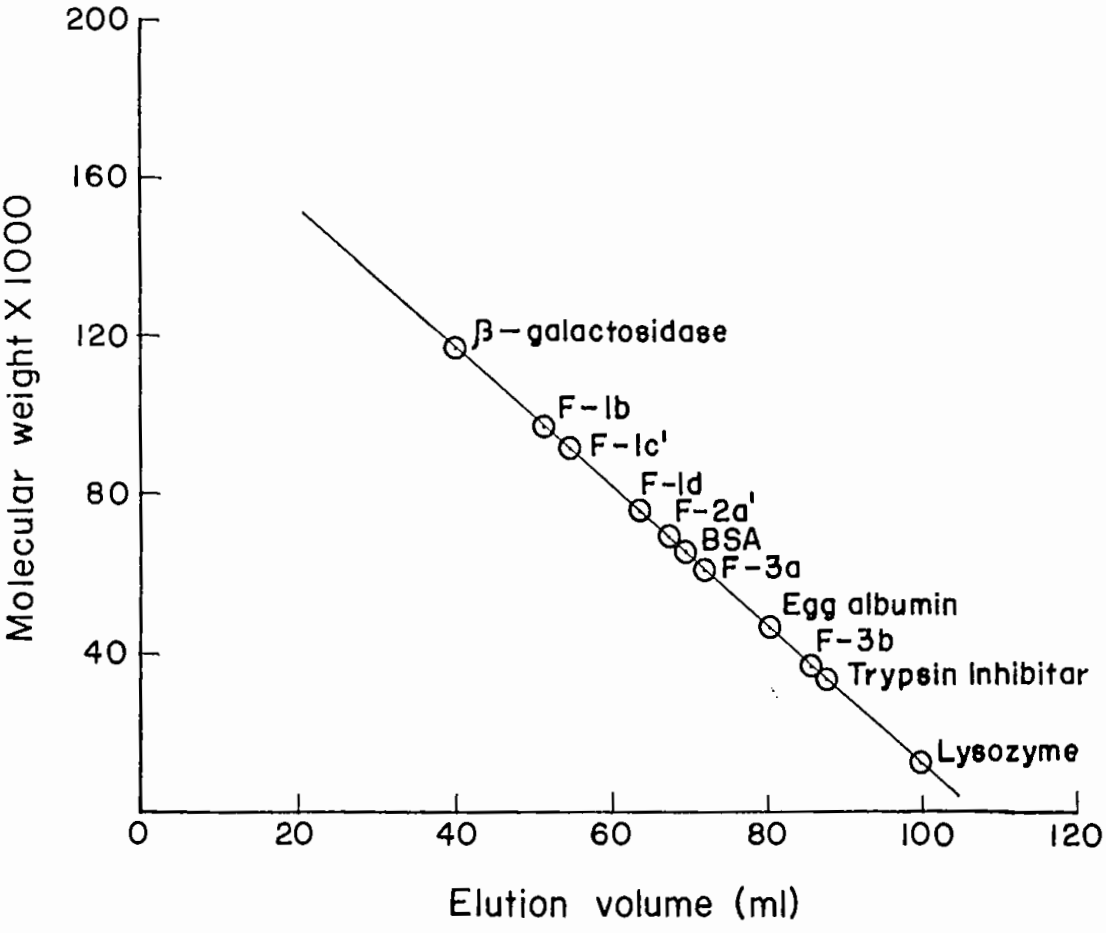


Fig 3.15. Standard curve for the determination of Molecular weight by Gel filtration on Sephadex G-150 . Size of the column (2.5 X 100 cm).

Flow rate 20 ml / hour. Buffer = Tris- HCl , pH- 8.4 .

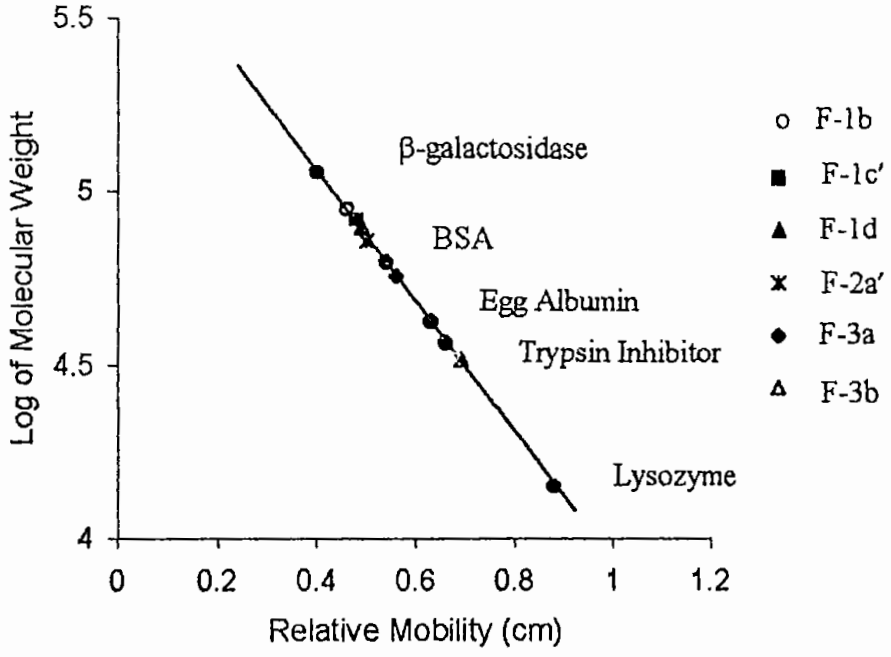


Fig. 3.16. Standard curve for the determination of molecular weight by SDS polyacrylamide slab gel electrophoresis.

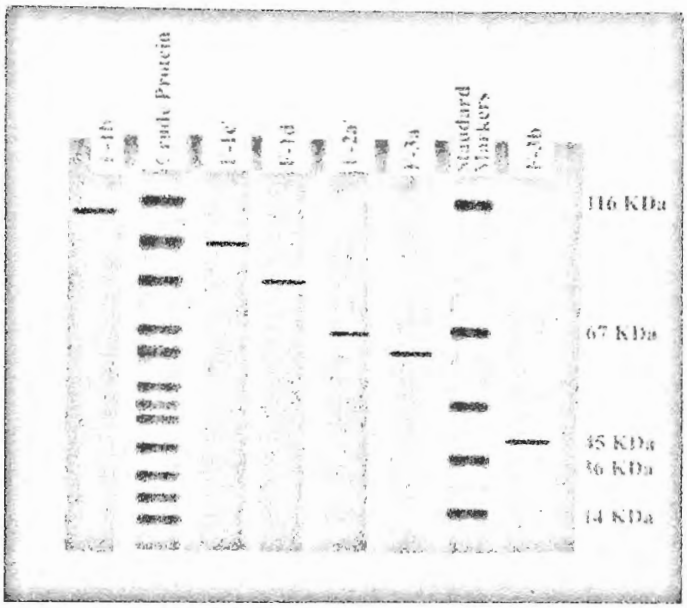


Fig. 3.17. SDS polyacrylamide slab gel electrophoretic pattern of fraction F-1C′, F-1d, F-2a′, F-3a′ and F-3b.

Staining reagent : 0.1% Coomassic brilliant blue : R – 250

Protein concentration : 75 μg

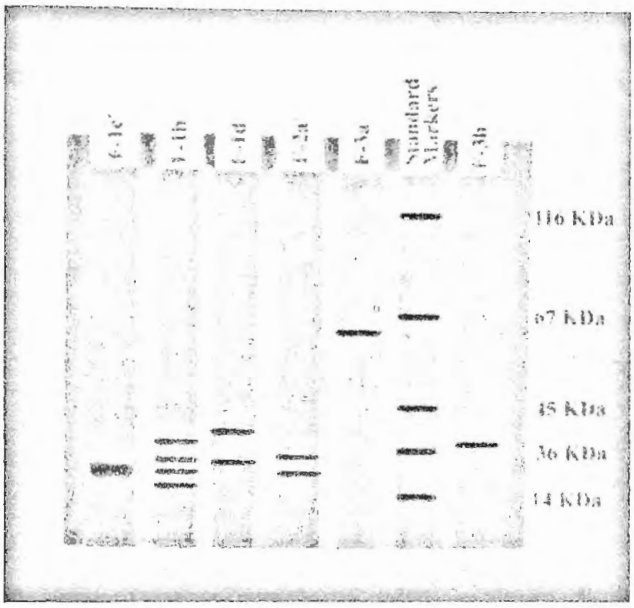


Fig. 3.18. SDS polyacrylamide slab gel electrophoretic pattern of F-1b, F-1C′, F-1d, F-2a′, F-3a′ and F-3b proteins for the determination of Molecular weight and subunit structure, in presence of 1% β-mercaptoethanol.

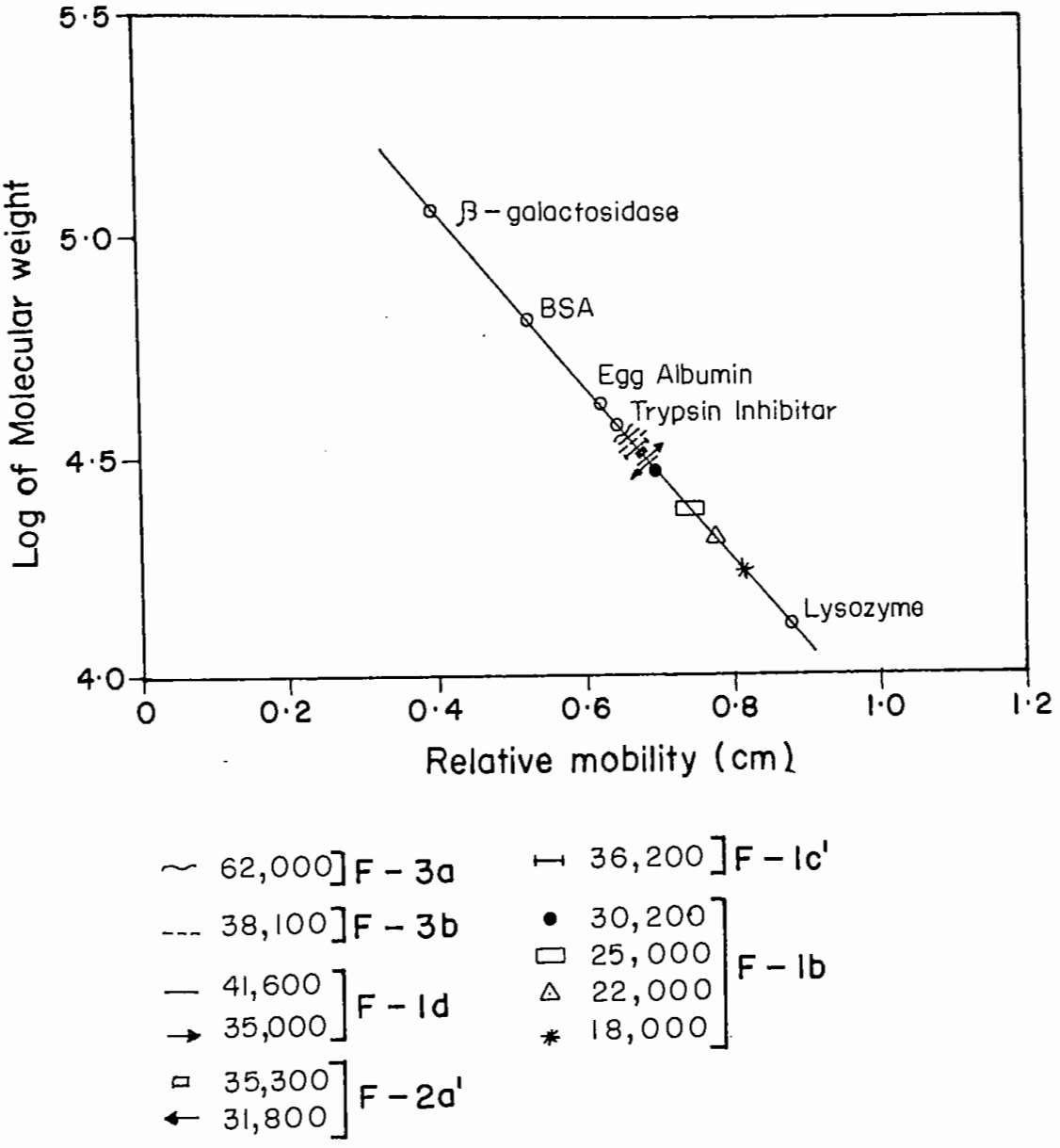


Fig. 3.19. Determination of purified proteins subunit by SDS-slab gel electrophoresis.

3.7.2. AFFINITY TO CONA – SEPHAROSE:

All the six lectins PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 were bound very tightly to ConA-sepharose at 4°C and the bound lectins were eluted from the column by 5mM phosphate buffer, pH-7.2 containing 0.2 M mannose. (Fig. 3.20)

3.7.3. HEMAGGLUTINATING ACTIVITIES OF THE LECTINS:

All the purified lectins agglutinated specifically albino rat red blood cells. The hemaglutination potency of rat red blood cells by the lectins were shown by photographic representation in Fig. 3.21, Fig. 3.22, Fig. 3.23, Fig. 3.24, Fig. 3.25 and Fig. 3.26 and the results were summarized in Table-3.3. The minimum protein concentration at 280 nm needed for visible agglutination was taken as minimum hemagglutination dose (MHD) and were found to be 9.3µg, 22.2 µg, 32.0µg, 12.8 µg, 9.7µg, and 22.5µg for F-1b (*Potamogeton nodosus* Lectin-1 i.e PNL-1), F-Ic′ (PNL-2), F-1d (PNL-3), F-2a′ (PNL-4), F-3a (PNL-5) and F-3b (PNL-6) respectively.

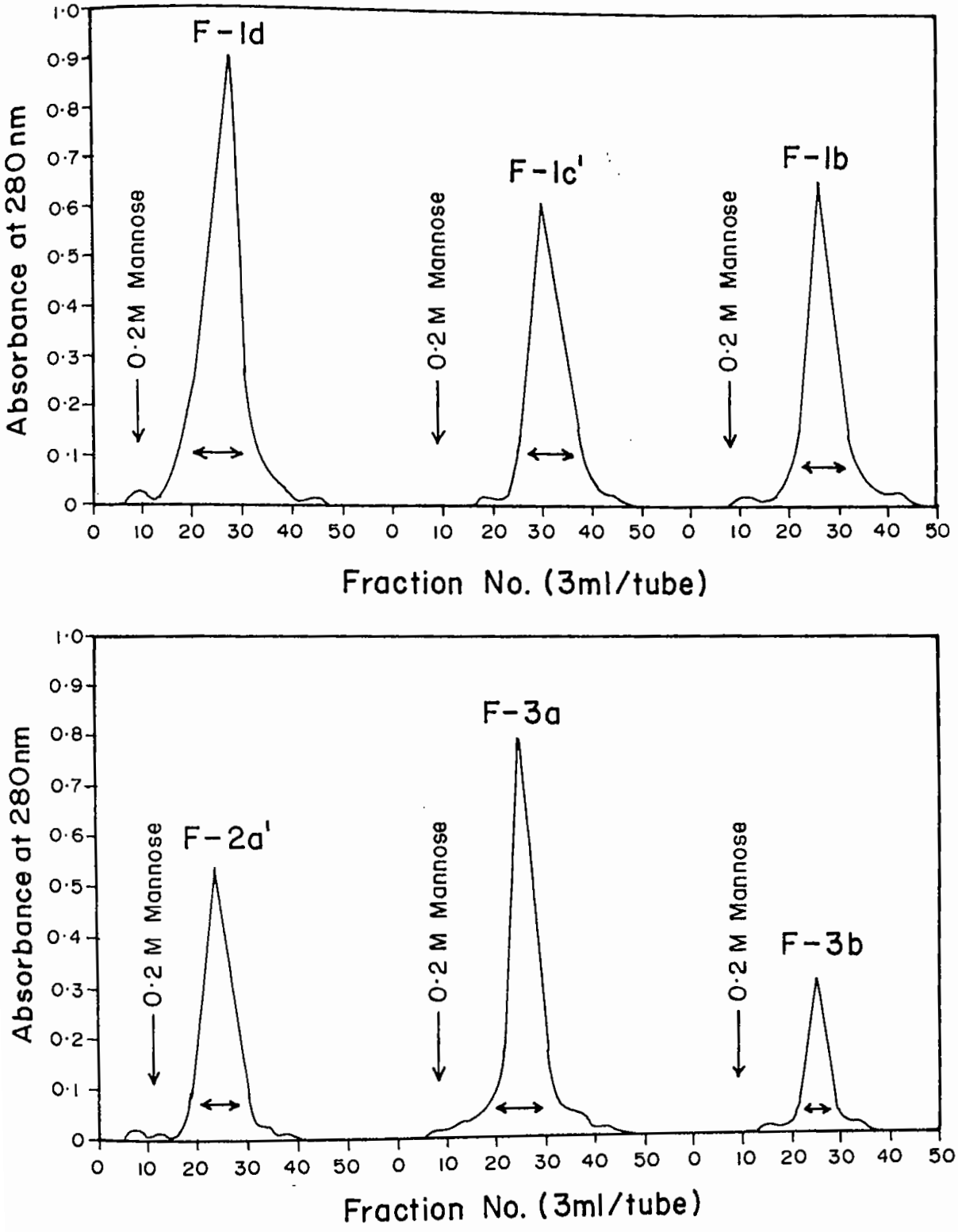


Fig. 3.20. Affinity chromatography of lectins, PNL-3, PNL-2, PNL-1, PNL-4, PNL-5 and PNL-6 on Con-A Sepharose. The fractions were applied individually to the column (1.25 x 10 cm) pre-equilibrated with 5 mM phosphate buffer saline, pH 7.2 at 4°C and the absorbed proteins were eluted by the same buffer containing 0.2 M mannose Flow rate: 30ml/hr

Table 3.3. Hemagglutinating activities of PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 with 2% red blood cells from albino rat.

Protein samples	Absorbance at 280 nm	Concentration (mg / ml)	Degree of Hemagglutination
PNL-1	0.045	0.0419	3^+
	0.035	0.0326	2^+
	0.02	0.0186	1^+
	0.01	0.0093	±
PNL-2	0.072	0.0533	3^+
	0.06	0.0444	2^+
	0.042	0.0311	1^+
	0.03	0.0222	±
PNL-3	0.08	0.0640	3^+
	0.065	0.0520	2^+
	0.050	0.0400	1^+
	0.040	0.0320	±
PNL-4	0.050	0.0425	3^+
	0.042	0.0357	2^+
	0.025	0.0213	1^+
	0.015	0.0128	±
PNL-5	0.050	0.0485	3^+
	0.040	0.0388	2^+
	0.020	0.0194	1^+
	0.010	0.0097	±
PNL-6	0.07	0.0525	3^+
	0.06	0.0450	2^+
	0.042	0.0315	1^+
	0.03	0.0225	±

3^+ Indicates complete aggregation of all most 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 small aggregates.

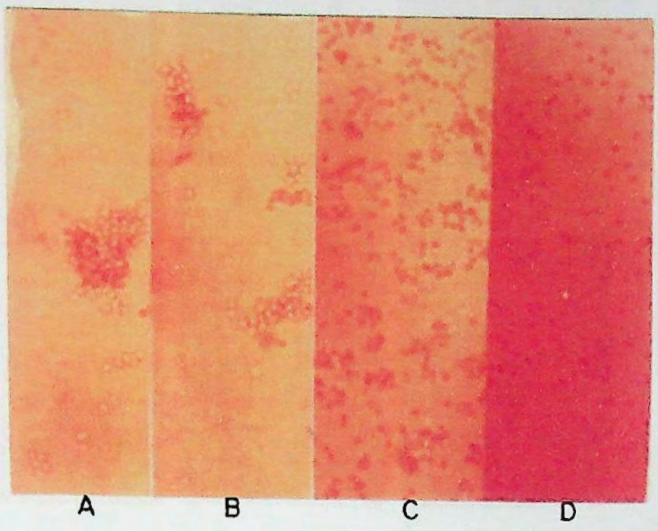


Fig. 3.21: Agglutination of albino rat red blood cells by PNL-1

A = 3^+; B = 2^+; C = 1^+ and D = control

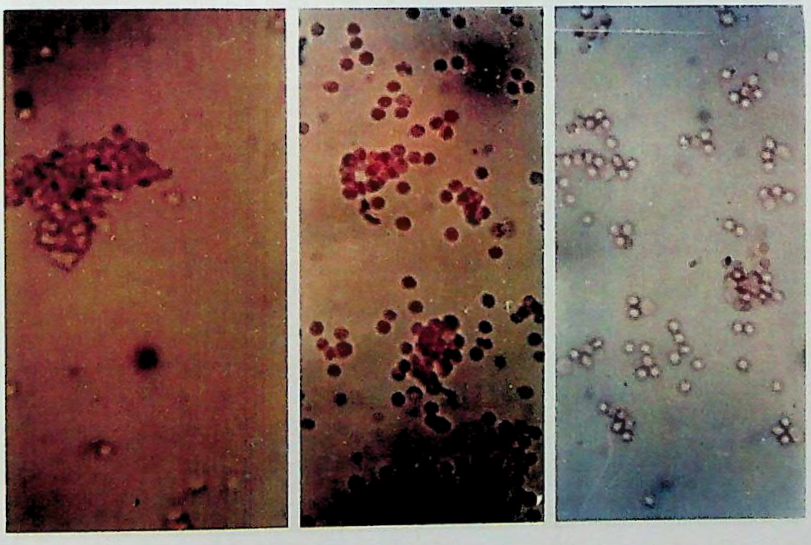
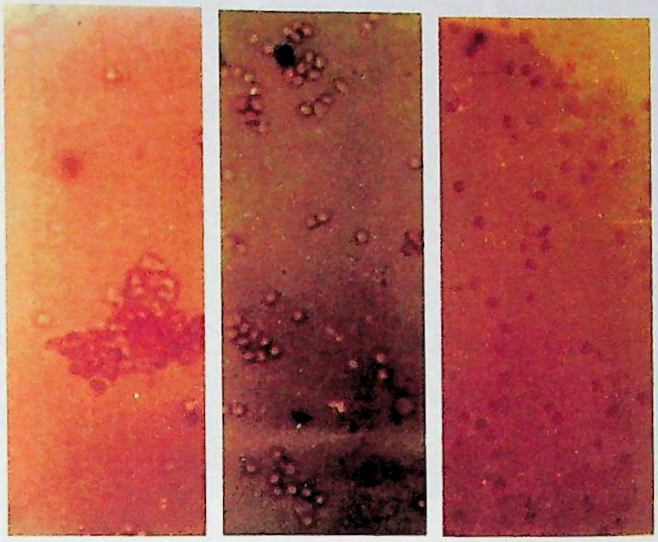


Fig. 3.22: Agglutination of albino rat red blood cells by PNL-2

A = 3^+; B = 2^+ and C = 1^+



A B C

Fig. 3.23: Agglutination Potency of albino rat red blood cells by PNL-3

$A = 3^+$; $B = 2^+$ and $C = 1^+$

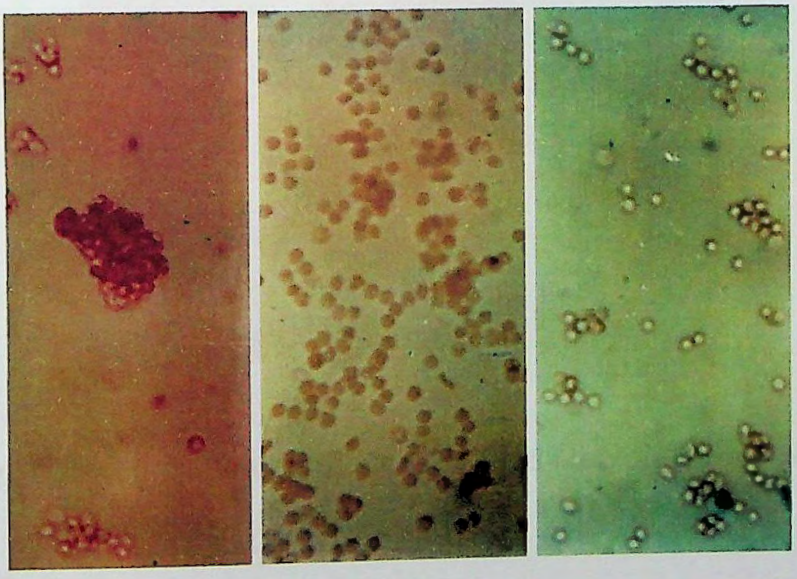


Fig. 3.24: Agglutination of albino rat red blood cells by PNL-4

$A = 3^+$; $B = 2^+$ and $C = 1^+$

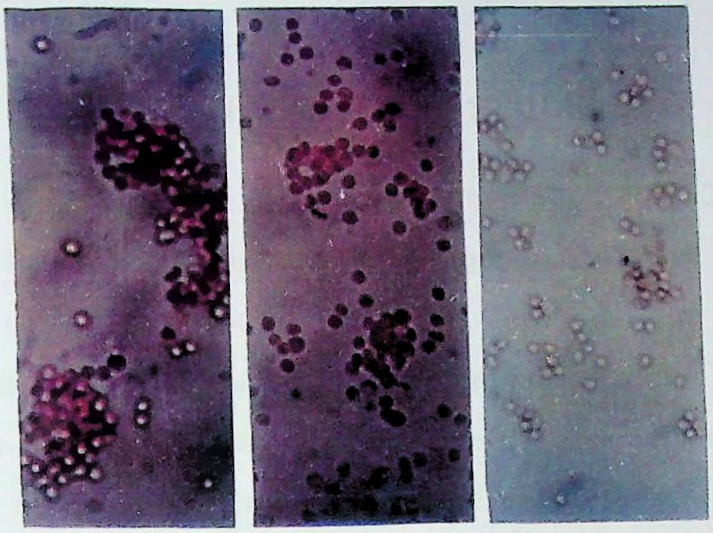


Fig. 2.25: Agglutination of albino rat red blood cells by PNL-5

$A = 3^+$; $B = 2^+$ and $C = 1^+$

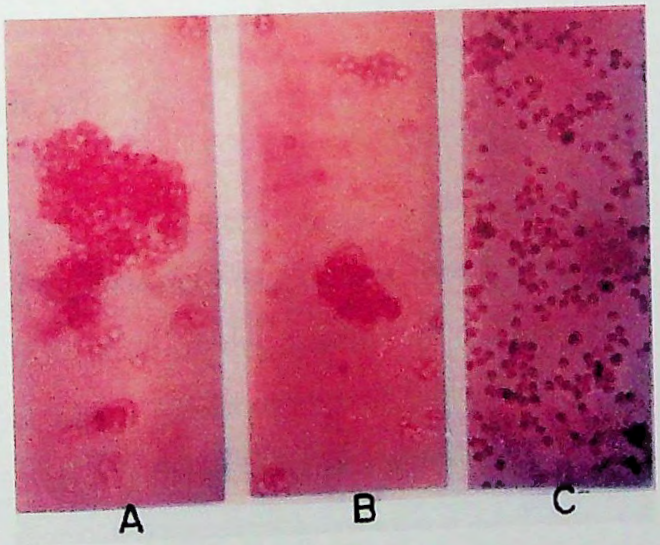


Fig. 2.26: Agglutination of albino rat red blood cells by PNL-6

$A = 3^+$; $B = 2^+$ 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.

3.7.4. HEMAGGLUTINATION-INHIBITION STUDIES:

Hemagglutination inhibition of rat red blood cells by PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 were performed in presence of different sugars and the results of the hemagglutination inhibition test of the *Potamogeton nodosus* rootstock lectins with haptenic sugars are presented in Table-3.4. It was evident from the result that mannose methyl α-D-mannopyranoside and β-D-mannopyranoside are the most potent inhibitors for all the six lectins.

Table 3.4. Hemagglutination-inhibition assay of *Potamogeton nodosus* rootstock lectins by different sugars.

Proteins	Sugar	Conc. (mM)	Inhibition
PNL-1	D-glucose	120	NI
	D-mannose	30	I
	D-galactose	120	NI
	D-Fructose	120	NI
	N-acetyl-D-galactosamine	120	NI
	Methyl-α-D-galactopyranoside	120	NI
	Methyl-β -D-galactopyranoside	120	NI
	Methyl-α-D-mannopyranoside	50	I
	Methyl-β-D- mannopyranoside	45	I
	Methyl-α-D- glucopyranoside	120	NI
PNL-2	D-glucose	120	NI
	D-mannose	30	I
	D-galactose	120	NI
	D-Fructose	120	NI
	N-acetyl-D-galactosamine	120	NI
	Methyl-α-D-galactopyranoside	120	NI
	Methyl-β -D-galactopyranoside	120	NI
	Methyl-α-D-mannopyranoside	50	I
	Methyl-β-D- mannopyranoside	45	I
	Methyl-α-D- glucopyranoside	120	NI

NI = No inhibition I = Inhibition

Proteins	Sugar	Conc.(mM)	Inhibition
PNL-3	D-glucose	40	I
	D-mannose	30	I
	D-galactose	120	NI
	D-Fructose	120	NI
	N-acetyl-D-galactosamine	120	NI
	Methyl-α-D-galactopyranoside	120	NI
	Methyl-β -D-galactopyranoside	120	NI
	Methyl-α-D-mannopyranoside	35	I
	Methyl-β-D- mannopyranoside	45	I
	Methyl-α-D- glucopyranoside	35	I
PNL-4	D-glucose	120	NI
	D-mannose	25	I
	D-galactose	120	NI
	D-Fructose	120	NI
	N-acetyl-D-galactosamine	120	NI
	Methyl-α-D-galactopyranoside	120	NI
	Methyl-β -D-galactopyranoside	120	NI
	Methyl-α-D-mannopyranoside	20	I
	Methyl-β-D- mannopyranoside	25	I
	Methyl-α-D- glucopyranoside	120	NI

NI = No inhibition I = Inhibition

Proteins	Sugar	Conc.(mM)	Inhibition
PNL-5	D-glucose	120	NI
	D-mannose	35	I
	D-galactose	120	NI
	D-Fructose	100	NI
	N-acetyl-D-glucosamine	120	NI
	Methyl-α-D-galactopyranoside	120	NI
	Methyl-β -D-galactopyranoside	120	NI
	D-glucoseamine-HCl	120	NI
	Methyl-α-D- glucopyranoside	120	NI
	Methyl-α-D-mannopyranoside	45	I
PNL-6	D-glucose	120	NI
	D-mannose	40	I
	D-galactose	120	NI
	D-Fructose	120	NI
	N-acetyl-D-glucosamine	120	NI
	Methyl-α-D-galactopyranoside	30	NI
	Methyl-β -D-galactopyranoside	65	NI
	D-glucoseamine-HCl	120	NI
	Methyl-α-D- glucopyranoside	120	NI
	Methyl-α-D-mannopyranoside	40	I

NI = No inhibition I = Inhibition,

3.7.5. ULTRAVIOLET ABSORPTION SPECTRA OF THE PURIFIED LECTINS

The purified lectins PNL-1, PNL-2, PNL-3, PNL-4 PNL-5 and PNL-6 in aqueous solution gave absorption maxima around 278, 280, 280, 278, 280 and 276 nm and minima around 242, 248, 240, 244, 250 and 244 nm respectively (Fig. 3.27).

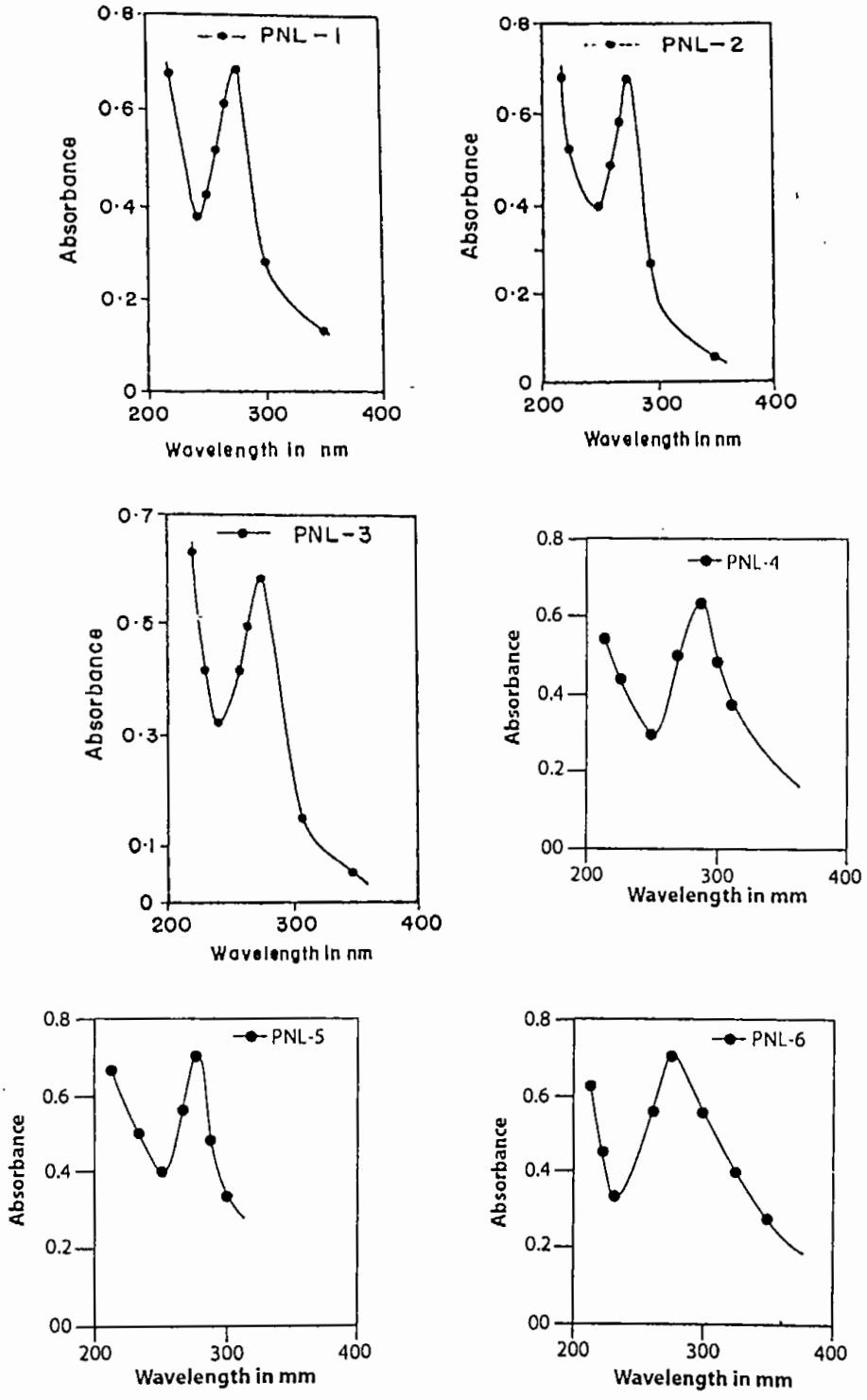


Fig. 3.27. Ultraviolet absorption spectra of the lectins.

3.7.6. CONCENTRATION OF PROTEIN:

The absorbance of 1.0 at 280 nm for PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 were found to be equal to 0.93, 0.74, 0.8, 0.85, 0.97 and 0.75 mg of proteins, respectively, by the Lowry methods.

Table 3.5. Optical density (O.D) and protein concentration relationship of the lectins.

Proteins	O.D. of proteins at 280 nm	Amount of protein obtained by Lowry method (mg)
PNL-1	1.0	0.93
PNL-2	1.0	0.74
PNL-3	1.0	0.8
PNL-4	1.0	0.85
PNL-5	1.0	0.97
PNL-6	1.0	0.75

3.7.7. Glycoprotein Test and sugar content

The purified lectins, PNL-1, PNL-2, PNL-3, PNL-4, PNL-5, and PNL-6 gave yellow orange color in the presence of phenol-sulfuric acid , indicating that the lectins contained sugar i.e. the proteins are glycoprotein. The presence of sugars in the proteins were also confirmed by periodic acid schiff (PAS) staining method as described by (Khomei Yanagi *et al.*, 1990). It was found that all the proteins gave pinkish red band or polyacrylamide slab gel, when gels were stained with PAS reagent after electrophoresis (Fig: 3.28). The percentage of neutral sugar present in the glycoproteins, PNL-1, PNL-2, PNL-3, PNL-4, PNL-5, and PNL-6 were found to be 3.22%, 4.0%, 3.75%, 3.72%, 3.4% and 3.10% respectively.

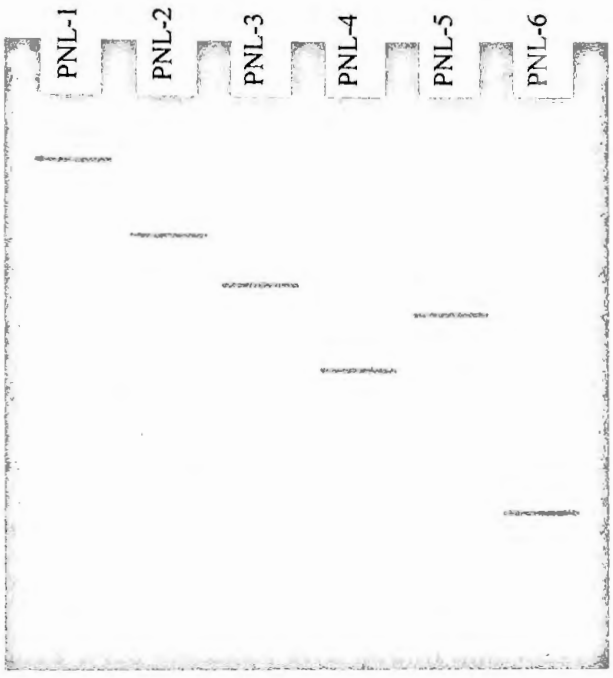


Fig. 3.28. Polyacrylamide slab gel electrophoretic pattern of PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 lectins on 7.5% gel after staining with periodic acid schiff's (PAS) reagent.

3.7.8. COMPARATIVE CYTOTOXICITY STUDY OF SIX BIOACTIVE LECTINS PURIFIED FROM PONDWEED (*Potamogeton nodosus* Poir) ROOTSTOCK ON BRINE SHRIMP:

All the six lectins showed a significant cytotoxic effects in the brine shrimp lethality bioassay (Table-3.6). Mortality of the nauplii was noticed in the experimental groups while at the same time the control group remained unchanged. The number of survived nauplii in each vial was counted and the results were noted. From these data the percent mortality of the brine shrimp was calculated for every concentration of each sample. The mortality rate of brine shrimp nauplii was found to increase with the increase in concentration of sample and a plot of log concentration versus percent mortality gave an almost linear correlation (Fig. 3.29). The LD-50 (concentration/dose at which 50% mortality of the nauplii occurs) values were obtained after probit analysis and the values for the lectins, PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 were found to be 10.76, 7.03, 17.25, 10.52, 19.61 and 20.10 μg/mL respectively.

Table 3.6. Brine shrimp lethality bioassay of lectins purified from pondweeds.

Sample	dose	log dose	Mortality%	corr%	emp probit	expt probit	wrk probit	weight	final probit
PNL-1	1.6	0.2041179	20	20	4.16	4.135998	4.17	47.1	4.139904
	3.2	0.5051448	28	28	4.42	4.452999	4.42	55.80	4.45275
	6.4	0.8061716	40	40	4.75	4.77	4.74	61.6	4.765597
	12.8	1.107199	55	55	5.13	5.087	5.125	63.7	5.078443
	25.6	1.408225	65	65	5.39	5.404001	5.375	60.1	5.39129
PNL-2	1.6	0.2041179	30	30	4.48	4.398	4.49	53.2	4.396008
	3.2	0.5051448	35	35	4.61	4.681	4.605	60.1	4.678851
	6.4	0.8061716	44	44	4.85	4.964	4.84	63.4	4.961695
	12.8	1.107199	64	64	5.36	5.247001	5.384	62.7	5.244539
	25.6	1.408225	70	70	5.52	5.530001	5.5	58.1	5.527382
PNL-3	1.6	0.2041179	20	20	4.16	4.126	4.17	47.1	4.130446
	3.2	0.5051448	26	26	4.36	4.381	4.362	53.2	4.383928
	6.4	0.8061716	35	35	4.61	4.636	4.605	60.1	4.637411
	12.8	1.107199	45	45	4.87	4.891001	4.890	62.7	4.890893
	25.6	1.408225	57	57	5.18	5.146001	5.165	63.4	5.144376
PNL-4	1.6	0.2041179	25	25	4.33	4.234	4.32	50.30	4.217438
	3.2	0.5051448	30	30	4.48	5.519	4.46	58.1	4.505436
	6.4	0.8061716	38	38	4.69	4.804	4.708	62.7	4.793434
	12.8	1.107199	52	52	5.05	5.089	5.05	63.7	5.081432
	25.6	1.408225	68	68	5.47	5.374	5.448	61.6	5.36943
PNL-5	1.6	0.2041179	15	15	3.96	4.02	3.955	43.9	4.022636
	3.2	0.5051448	25	25	4.33	4.292	4.32	50.30	4.292971
	6.4	0.8061716	35	35	4.61	4.564	4.6	58.1	4.563306
	12.8	1.107199	45	45	4.87	4.836001	4.890	62.7	4.833641
	25.6	1.408225	52	52	5.05	5.108001	5.04	63.4	5.103976
PNL-6	1.6	0.2041179	25	25	4.33	4.414	4.33	55.80	4.411319
	3.2	0.5051448	36	36	4.64	4.574	4.628	58.1	4.572556
	6.4	0.8061716	42	42	4.80	4.734	4.792	61.6	4.733793
	12.8	1.107199	46	46	4.90	4.894	4.916	62.7	4.895029
	25.6	1.408225	50	50	5.00	5.054001	5.00	63.7	5.056267

Table 3.7. Toxicity levels of the lectins purified from pondweeds.

Sample	Regression Equation	Chi-Square Value	LOG LD-50	LD-50	95% Conf. limits
PNL-1	Y = 3.927771 + 1.039265 X	0.2967148	1.031719	10.75769	8.118416 TO 14.25498
PNL-2	Y = 4.20422 + 0.9395951 X	2.999058	0.8469398	7.029748	8.118416 TO 14.25498
PNL-3	Y = 3.958566 + 0.8420597 X	0.1901894	1.23677	17.24924	11.20608 TO 26.55132
PNL-4	Y = 4.022155 + 0.9567182 X	1.550083	1.022083	10.52162	7.791081 TO 14.20913
PNL-5	Y = 3.839329 + 0.8980431 X	0.7745476	1.292445	19.60852	12.71328 TO 30.24348
PNL-6	Y = 4.301988 + 0.5356231 X	0.9862089	1.303177	20.09912	9.684488 TO 41.71359
Gallic Acid (Reference)		1.25		4.53	3.33 TO 6.15

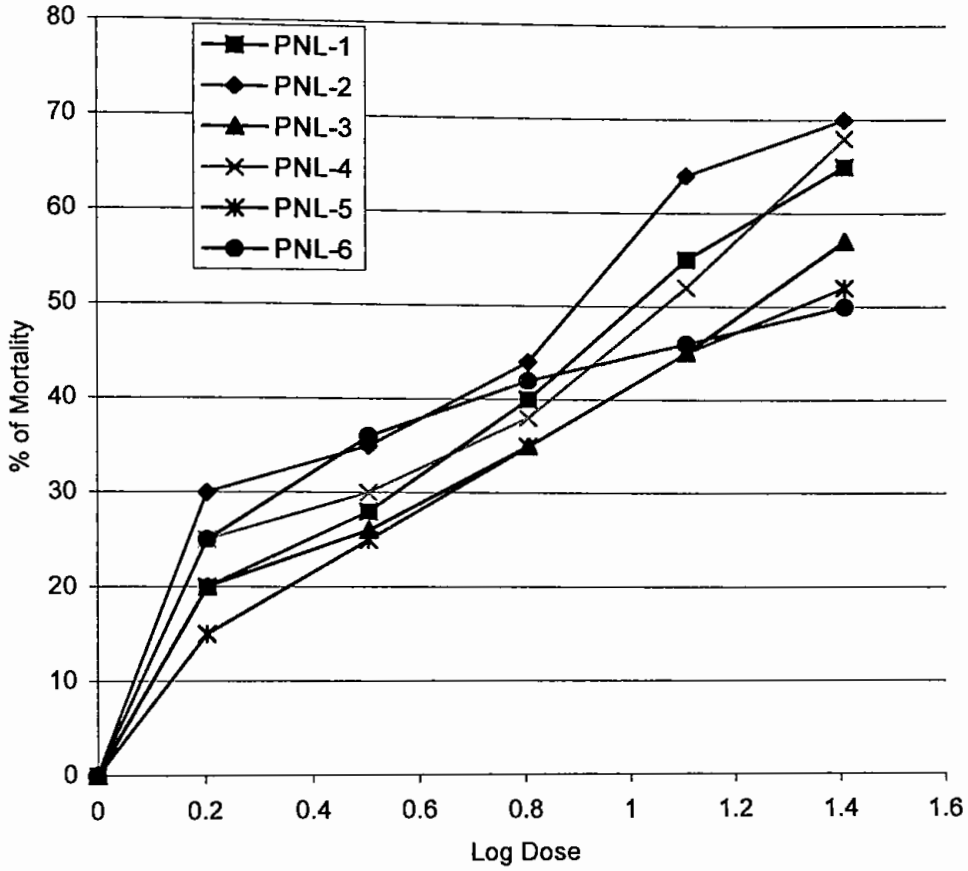


Fig. 3.29. Determination of LD-50 from Log Conc. response curve.

3.8.9. IDENTIFICATION OF REDUCING SUGAR PRESENT IN THE PURIFIED PROTEIN BY THIN LAYER CHROMATOGRAPHY (TLC).

The sugar composition of the lectins, as determined by one dimensional thin layer chromatography, were identified to be D-galactose for PNL-1 and PNL-6 while mannose for PNL-2, PNL-3 and PNL-4 and D-glucose for PNL-5 (Table 3.8).

Table 3.8. Data of the R_f values obtained by TLC examination of the sugars from hydrolyzed protein samples and standard sugars.

Standard sugars & protein samples	Distance traveled by the solvent system	Distance traveled by the standard sugar & protein	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
D-ribose	13.9	9.40	0.676
Protein :			
PNL-1	13.9	10.38	0.747
PNL-2	13.9	8.87	0.640
PNL-3	13.9	8.76	0.630
PNL-4	13.9	8.82	0.634
PNL-5	13.9	9.29	0.668
PNL-6	13.9	10.40	0.748

3.9. DISCUSSION:

Six proteins have been isolated and purified from the crude protein extract of the Rootstock of *Potamogeton nodosus* with a purification fold of about 83.19, as compared to that of the crude protein extract. The homogeneity of the purified protein were confirmed by polyacrylamide slab gel electrophoresis (7.5%) as they gave single band on the gel (Fig. 3.10 & Fig. 3.11). All these proteins are glycoproteins in nature as they gave orange yellow color in the presence of phenol sulfuric acid (Dubois *et al.,* 1956). Which showed that rootstock protein contained about 3-4% neutral sugar. So, the carbohydrate content of these purified proteins are very much similar to must of the plant lectins (around 5%). The presence of sugar in the lectins was further confirmed by observing the pinkish red band produced on SDS-polyacrylamide slab gel when stained with periodic acid Schiff's staining reagent (Khomei Yanagi *et al.,* 1990) after electrophoresis (Fig. 3.28).

The agglutinations of rat red blood cells by the proteins are inhibited specifically in the presence of mannose and mannose containing saccharide, indicating that all the purified proteins from rootstock of *Potamogeton nodosus* belong to the class of plant lectins which are specific for mannose and mannose containing sugar. These findings were further supported by the findings that all the six lectins showed binding affinity to Con-A sepharose and the lectin Concanavalin A (ConA-sepharose) is also a mannose / glucose specific lectin (Agarwal and Gold Stein 1967).

It is interesting to note that so many lectins of similar sugar specificity are purified from the same sources in this study.

Each of the lectins were found to migrate as a single band with different mobilities on polyacrylamide gels but in the presence of SDS, all the six lectins moved as a single band except PNL-1. Further, in the presence of SDS, PNL-1 was transformed into four subunits of different molecular masses of 30,200; 25,000; 22,000 and 18,000 indicating that the lectin PNL-1 is tetramer is nature and its subunits are held together by nonionic hydrophobic interaction. PNL-2 was transformed into single band corresponding to molecular weight of 36,200 in the presence of SDS and β-mercaptoethanol indicating that this protein contains four subunits of equal size which are held together by S-S bond, but PNL-3 and PNL-4 are transformed into two subunits of different molecular masses under same conditions indicating that these proteins are dimer which are held together by S-S bonds. On the other hand, PNL-5 and PNL-6 gave single band on the gel in the presence and absence of β-mercaptoethanol indicating that the proteins are monomer. The lectins purified from plant sources often contained mostly four sub-units of two identical pairs, viz., Indian bean (*Dolichos lablab* L.) (*Guruan et al.,* 1983) *Arbus precatorius* (Absar and Funatsu, 1984) and *Ricinus comminis* agglutnin (Olsnes *et al.,* 1974) and very few contained four subunits of identical molecular weight, viz., *Phaseolus vulgaris* (Itoh *et al.,* 1980). Some lectins are dimer with 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 from different sources such as *Abrus precatorius* (Absar and Funatsu, 1984) and Ricinus comminis (Olsnes *et al.,* 1974) etc. lectins from Rootstock of *Potamogeton nodosus* are also cytotoxic in nature which was also confirmed from the brine shrimp lethality bioassay.

In conclusion, the purified lectins PNL-1, PNL -2, PNL-3, PNL-4, PNL-5 and PNL-6 besides being specific for rat red blood cells agglutinations, can be added as an addition of members to the list of the purified member of mannose specific lectins.

CHAPTER FOUR

Effect of Physico-chemical Agents on the Hemagglutination Activities *Potamogeton nodosus* Root Stocks

4.1. INTRODUCTION:

The three dimensional 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 other denaturing solution and a number of other chemicals.

In structural studies of proteins it is often necessary to establish conditions for reversible denaturation . The choice of denaturating condition depends on the stability of the protein of interest. Among the techniques used for reversible denaturation are lowering of the pH (Itano *et al.* 1958), freezing and thawing in concentrated salt (Market, C.L. 1963), and adding denaturants such as urea and Guanidine-HCl (Chilson *et al.* 1964 : and 1965 : Meighen and Schachman 1970).

PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 are glycoproteins purified from rootstocks of *potamogeton nodosus* , specifically agglutinate rat red blood cells and may be used to perform many other biological activities.

In the present study, all the above mentioned lectin have been subjected to various physical and chemical treatments, and their effects on the hemagglutinating activities were observed .The study is expected to provide important information regarding some of the physico-chemical properties such as pH stability, thermal stability, the stability of the lectin towards denaturing agents etc. The experimental results also give indications in establishing conditions for chemical modification which in turn, are expected to be helpful in understanding the relationship between structure and function .

4.2. MATERIALS AND METHODS

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

4.2.1. HEMAGGLUTINATING ACTIVITY:

The hemagglutinating activity was performed by using 2% albino rat red blood cells according to the method of Lin *et al.* (1981) as described earlier in Chapter 3.

4.2.2. DETERMINATION OF PH STABILITY:

The pH stability of PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 were examined by incubating the lectin solutions (0.25-0.35%) in 50 mM respective buffers possessing pH ranges from 2.0 - 10.5 for 10 hr at 28°C. The hemagglutinating activity retained was determined after dialysis against 5 mM phosphate buffer saline (PBS), pH 7.2 for 18 hr at 4°C.

4.2.3. DETERMINATION OF HEAT STABILITY:

The lectin solutions (0.2-0.3%) in 5 mM phosphate buffer saline, pH 7.2 were heated at various temperature for 1hr in a temperature controlled water bath. The hemagglutinating activity of all the lectins was determined after cooling the heated lectin solutions in an ice bath and diluting with the same buffer.

4.2.4. TREATMENT WITH ACETIC ACID:

To the aqueous lectin solutions (0.97mg / ml), were added acetic acid at different concentrations. After an incubation period of 1 hr at 4°C, the lectin solutions were dialyzed against 5 mM phosphate buffer saline, pH 7.2 for 12 hr at 4°C and the hemagglutinating activity was determined.

4.2.5. TREATMENT WITH UREA:

To the lectin solutions (0.97mg / ml in 10 mM Tris- HCl buffer, pH –8.2), were added solid urea to the concentrations of 0, 2, 4, 6, 8 and 10 M. After incubation at 15°C for 12 hr, the solutions were dialyzed against 5 mM phosphate buffer saline, pH 7.2 for 12 hr at 4°C to remove urea and the hemagglutinating activity was determined.

4.2.6. TREATMENT WITH GUANIDINE-HCL

To the lectin solutions (0.97mg / ml in 10 mM Tris- HCl buffer, pH –8.2), were added solid guanidine-HCl to the concentrations of 0, 0.5, 1, 2, 4 and 6M . After incubation at 20°C for 12 hr. the solutions were dialyzed against 5 mM phosphate buffer saline, pH 7.2 for 12 hours at 4°C to remove the reagent and the hemagglutinating activity was determined.

4.2.7. TREATMENT WITH VARIOUS SALTS:

The metallic salts of different concentrations were added to the lectin solutions (0.97 mg / ml in 10 mM Tris- HCl buffer, pH –7.2) and incubated for 30 minutes at room temperature. The hemagglutinating activity was assayed as described before. In this experimental procedure deionized water was used .

RESULT

4.3.1. FFECT OF pH:

As given in the table-8, the hemagglutinating activities of all the lectins were affected markedly with the changes of pH. The activities of the lectins were found to higher in the neutral pH ranges and the lectins, PNL-1, PNL-2, and PNL-3 gave maximum activities in the pH region of 6.5-7.2, 6.5-8.0 and 7.2 respectively while PNL-4, PNL-5 and PNL-6 showed maximum activities in the pH region of 6.5-7.2 only. Beyond these pH ranges, the activities of lectins were decreased gradually both at the acidic as well as alkaline pH and the activities of the lectins were abolished almost completely at pH 2.0 and at pH 10.

Table-4.1. Hemagglutinating activities of the lectins at different pH values.

pH (Buffer composition)	Relative hemagglutinating activity (%)					
	PNL-1	PNL-2	PNL-3	PNL-4	PNL-5	PNL-6
2.0 (KCl- HCl)	5	5	0	5	0	0
3.0 (AcONa - HCl)	20	15	10	20	5	5
4.0 (AcONa – CH_3COOH)	30	30	25	35	10	15
5.5 (NaH_2PO_4 - Na_2HPO_4)	35	45	40	50	20	25
6.0 (NaH_2PO_4 - Na_2HPO_4)	70	60	75	80	70	75
6.5 (NaH_2PO_4 - Na_2HPO_4)	90	80	100	100	80	85
7.2 (NaH_2PO_4 - Na_2HPO_4)	100	100	70	90	100	100
8.0 (NaH_2PO_4 - Na_2HPO_4)	75-80	90	30	70	60	55
9.0 (NaH_2PO_4 - Na_2HPO_4)	35	40	10	30	20	15
10.0 (NaH_2PO_4 - Na_2HPO_4)	10	5-10	0	10	10	5

4.3.2. FFECT OF TEMPERATURE:

As shown in Table-4.2, the activities of the purified lectins were also markedly affected with the changes of temperatture. The lectins, PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 gave maximum activities around 20-35^{0}C. With further rise of temperature, the activities of lectins decreased gradually and all the six lectins lost their activities completely at 70^{0}C.

Table-4.2. Heat stability of purified lectins.

Temperature (^{0}C)	Relative hemagglutinating activity (%)					
	PNL-1	PNL-2	PNL-3	PNL-4	PNL-5	PNL-6
20	100	100	100	100	100	100
35	100	100	90	95	100	100
40	75	65	50	60	75	70
50	35	30	25	40	35	30
60	20	20	10	25	20	15
70	0	0	0	0	0	0

4.3.3. EFFECT OF ACETIC ACID

The effects of acetic acid on the hemagglutinating activities of PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 are given in Table-4.3.

As shown in the table, the lectins PNL-1, PNL-2, PNL-5 and PNL-6 retained almost full hemagglutnating activity even after treatment with 0.5% acetic acid. The activities of the lectins were decreased rapidly with further increase of acetic acid concentration and the lectins, lost their activities almost completely in the presence of 10% acetic acid.

Table-4.3. Effect of acetic acid on the hemagglutinating activities of purified lectins.

Concentration of acetic acid (%)	Relative hemagglutinating activity (%)					
	PNL-1	PNL-2	PNL-3	PNL-4	PNL-5	PNL-6
0.0	100	100	100	100	100	100
0.5	100	100	90-95	95	100	100
1.0	80	70	75	80	80	75
2.5	40	40	30	35	40	45
5.0	30	25	10	15	30	25
10.0	10	0	0	0	10	5

4.3.4. EFFECT OF UREA

As shown in Table-4.4, the hemagglutinating activities of PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 were decreased rapidly after treatment with increasing concentration of urea and all the lectins lost their activities completely after treatment. with 8M urea.

Table-4.4. Effect of Urea on the hemagglutinating activities of the lectins.

Concentration of Urea (molar)	Relative hemagglutinating activity (%)					
	PNL-1	PNL-2	PNL-3	PNL-4	PNL-5	PNL-6
0	100	100	100	100	100	100
1	100	95	90	90	100	90
2	75-80	80	60	75	75	65
4	50-55	50	30	40	50	35
6	20	15	0	10	20	0
8	0	0	0	0	0	0

4.3.5. EFFECT OF GUANIDINE HYDROCHLORIDE:

As presented in Table 4.5, the hemagglutinating activities of PNL-1, PNL-2, PNL-3, PNL-4, PNL-5 and PNL-6 were also decreased markedly after treatment with guanidine hydrochloride and the present data indicated that the lectins retained about 30-50% activities after treatment with

2M Guanidine-HCl. The activities of the lectins were decreased further with further rise in guanidine-HCl concentration and all lectins lost their activities completely after treatment with 6M guanidine-hydrochloride.

Table-4.5. Effect of Guanidine-HCl on the hemagglutinating activities of the purified lectins.

Concentration of guanidine-HCl (molar)	Relative hemagglutinating activity (%)					
	PNL-1	PNL-2	PNL-3	PNL-4	PNL-5	PNL-6
0.0	100	100	100	100	100	100
0.5	90	80	80	85-90	85	80
1.0	70	70	50	55	70	55
2.0	50	50	30	45	50	30
4.0	30	20	10	25	20	9
6.0	0	0	0	0	0	0

4.3.6. EFFECTS OF METALLIC SALTS

Table-4.6 represents the effect of various metal ions and salts on the hemagglutinating activities of the purified lectins. From the Table, it is evident that the activities of all the lectins were abolished almost completely in the presence of 50 mM EDTA while in the presence of metallic salts such as Ca^{2+} and Cu^{2+}, the activities of the lectins were increased. On the other hand, the metallic salts of Na^{+}

and K^+ produced significant inhibitory effect on the activities of the lectins. Further, the metallic salts of Mg^{2+} was found to be non inhibitory to PNL-1 and PNL-2 but produced moderate inhibitory effect on the activity of PNL-3, PNL-5 and PNL-6.

Table-4.6. Effects of various metal ions and salts on the activities of the lectins

Salt added	Concentration (mM)	Relative hemagglutinating activity (%)					
		PNL-1	PNL-2	PNL-3	PNL-4	PNL-5	PNL-6
None	---	100	100	100	100	100	100
EDTA	50	0	5	0	5	0	0
$MgCl_2$	50	100	100	70	110	80	85
	100	100	100	50	120	60	65
Na_2SO_4	50	80	60	60	75	50	45
	100	70	50	55	50	65	60
$CaCl_2$	50	130	110	105	115	130	115
	100	140	120	110	140	120	125
$CuCl_2$	50	110	120	125	105	100	100
	100	130	140	140	120	120	110
KCl	50	55	40	70	80	90	85
	100	40	30	60	55	85	80
$NaNO_3$	50	80	60	70	75	70	85
	100	70	50	60	55	60	75

DISCUSSION

The aim of this study is to determine the stability of lectins by using physical and chemical means.

From the findings of the effect of pH and temperature, it can be concluded that the activities of all the three lectins were remarkably influenced with the changes of temperature as well as pH. The decreased in activities in extreme acidic as well as alkaline pH values may be due to ionization of groups located at or near the active sites. Further the decrease in activities at higher temperatures might be due to denaturation or disorganization of the secondary and tertiary structure of the lectins. On the basis of stability, the lectins may be arranged in the following order:

The biological activities of the lectins were also followed after treatment with acetic acid. The results showed good correlation with those obtained by previous experiments, i.e. effect of pH and temperature. It can be suggested from the results of acetic acid treatment that the lectins, PNL-1 is slightly more stable than the other lectin. The decrease in activity after treatment with higher concentration of acetic acid might be due to denaturation or destruction of the native structure of the lectins. The hemagglutinating activities of the lectins were also investigated by using guanidine-HCl and urea as denaturants. It was found that PNL-1, PNL-3, PNL-4, PNL-5 are slightly more stable in urea and in guanidine-HCl than PNL-2 and PNL-6. In the presence of 6M urea, the activities of PNL-1, PNL-2, PNL-4, PNL-5 became 20%, 15%, 10% and 20% respectively while that of PNL-3 and PNL-6 abolished completely. Similarly in the presence of 4M guanidine-HCl, the activities of PNL-1, PNL-2, PNL-4 and PNL-5 became 30%, 20%, 25% and 20% respectively while that of PNL-3 and PNL-6 became 10% and 9% respectively.

PNL-1> PNL-3> PNL-4> PNL-5> PNL-2> PNL-6>.

The activities of all the lectins were enhanced significantly in the presence of metallic salts, Ca^{2+} and Cu^{2+} suggesting the involvement of some metallic ions necessary for the activities of the lictins. Remarkably, EDTA, a metal chelator inhibited the hemagglutinating activities of the lectins greatly. It might be revealed again from these results that the metal ions are essential for hemagglutinating activities of the lectins and the metal ions were released completely/partially from the lectins after treatment with EDTA. The inhibitory effect of EDTA on hemagglutinating activity have also been observed on lectins of other sources such as TM (Tora-mame) lectin, a lectin from *Phaseolus valgaris* seeds (Itoh,*et al.* 1980).

Many lectins have been reported to be metalloproteins (I.J. Goldstein and C.E.Hayes, 1978) and a part of the metals is necessary for activities such as hemagglutination (Takahashi *et al.* 1971; Paulova *et al.* 1971; Tunis M, 1965; Alford,R.H. 1970), polysaccharide precipitation (Paulova *et al.* 1971) and lymphocyte transformation (Takahashi *et al.* 1971 & Alford R. H. 1970). In *Potamogeton nodosus* rootstock's lectins, metal ions such as Cu^{2+} or Ca^{2+} may be present in low concentration and a part of the metals might have been removed from the lectin molecules during the purification steps. This possibility may be supported from the observation that the hemagglutinating activities increased to about 20-40% by the addition of Ca^{2+} and Cu^{2+} to the purified lectin (Table-13). It may be concluded from the present data that the purified rootstock's lectins might be used in active form for biological purposes in the pH ranges of 6.5-7.2 and at the temperature value of 20-35°C. Further the salts of Ca^{2+} and Cu^{2+} may included during the experimental process but the salts of Na^{+} and K^{+} might be excluded from the experimental works as they are inhibitory to the lectins.

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