Studies on some biologically important proteins from *Moringa oleifera* seeds.

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that this thesis entitled "Studies on some biologically important proteins from *Moringa oleifera* seeds" submitted to the University of Hyderabad by Mr. KIRAN KUMAR TEJAVATH for the degree of Doctor of Philosophy, is based on the studies carried out by him under my supervision. I declare to the best of my knowledge that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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DECLARATION

I hereby declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of Prof**. N. Siva Kumar**. I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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ABBREVIATIONS

BSA	Bovine serum albumin
CAZY	Carbohydrate-active enzymes
CD	Circular Dichroism
CM	Carboxy Methyl
Con A	Concanavalin A
DE-52	DiEthyl-52
DEPC	Diethyl pyrocarbonate
DLL-II	Galactose-specific lectin from seeds of *Dolichos lablab*
DMJM	Deoxymannojirimycin
DTT	Dithiothreitol
DVS	Divinyl sulfone
EDTA	Ethylene diamine tetraacetic acid
et al	et alii (Latin: and others)
Gal	Galactose
GC1A	Galactosyl C1 – Amine
Gdn–HCl	Guanidium Hydrochloride
GH	Glycoside hydrolase
Glu/Glc	Glucose
Glu2NI	Glucosyl Naphthyl Imine
Glu2SI	Glucosyl Salicilyl Imine
GNI	Galactosyl Naphthyl Imine
Gp%	Glycoprotein %
GSI	Galactosyl Salicilyl Imine
IgG	Immunoglobulin G
kDa	Kilo Dalton
Lac	Lactose
LC1A	Lactosyl C1 – Amine
LNI	Lactose Naphthyl Imine

LSI	Lactose Salicilyl Imine
MALDI	Matrix assited laser desorption ionisation
min	Minute
mL	Milli Litre
MNI	Mannosyl Naphthyl Imine
MOCP	*Moringa oleifera* coagulant protein
MoSL	*Moringa oleifera* Seed Lectin
MS	Mass Spectrometry
MW	Molecular Weight
NBS	N-Bromosuccinimide
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide Gel electrophoresis
PBS	Phosphate-buffered saline
pH	-log (H^+) concentration
PKM1	Periyakulam
PMF	Peptide mass Fingerprints
PMSF	Phenyl methyl sulfonyl fluoride
pNP	para nitro phenol
*p*NPG	*p*-nitrophenyl-α-D-Galactopyranoside.
*p*NPM	*p*-nitrophenyl-α-D-Mannopyranoside.
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
SW	Swainsonine
TBS	Tris-buffered saline
UV	Ultraviolet

CHAPTER 1

General introduction and scope of present investigation




General introduction

Moringa oleifera (*M.oleifera*)

Moringa oleifera Lam is a tree native to India and is a highly valued plant, distributed throughout tropics and subtropics. It belongs to the genus *Moringaceae*. A single genus with 14 known species, it is the most widely known and cultivated of these [Morton, 1991]. *M.oleifera* is a multipurpose tree with most of its parts (leaves, flowers, fruits, wood and seeds) having pharmacological and nutritional properties and it is often referred to as the 'miracle tree'. All parts of the *Moringa* tree have long been consumed by humans and are an important source of food.

According to Fuglie [1999] the many uses for *Moringa* include: alley cropping (biomass production), animal forage (leaves, seed-cake), biogas (leaves), domestic cleaning agent (leaves), blue dye (wood), fencing (trees), fertilizer (seed-cake), foliar nutrient (leaves), green manure (leaves), gum (trunks), honey- and sugar cane juice-clarifier (seeds), honey (nectar), medicine (all plant parts), ornamental plantings, biopesticide (leaves), pulp (wood), rope (bark), tannin for tanning hides (bark and gum), water purification (seeds). *Moringa* seed oil (yield 30-40% by weight), also known as Ben oil, is a sweet non-sticking, non-drying oil that resists rancidity. It has been used as a fine lubricant in delicate machines and in the cosmetics industry.

Over the past few decades, many reports have appeared in mainstream scientific journals describing its nutritional, phytochemical and pharmacological properties. Many studies [Jahn, 1988; Ndabigengesere *et al.,* 1995; Muyibi and Evison, 1996] have been done on the performance of *M.oleifera* seeds as a natural coagulant in water treatement. Its utility as a biological absorbent has also been extensively described [Sharma *et al.*, 2006]. Very recently different lectins have been purified from this plant which have the coagulant and insecticidal properties [Coelho *et al.*, 2009; Oliveira *et al.*, 2010]. This thesis focuses on some of the physiochemical properties of the coagulant protein thought to be the principle component in the water purification along with the antinutritional factor lectin and glycosidases and their relationship all of which are extracted from the seed kernel.

The genus ***Moringa*** *is* classified as follows:

Kingdom : Plantae

Phylum : Angiosperms

Class : Eudicots

Order : Brassicales

Family : *Moringaceae*

Genus : *Moringa oleifera*

Species : *Moringa oleifera* Lam

Variety : Periyakulam 1(PKM_1) [*The variety used in the present study*]

Moringa oleifera Lam, commonly referred to as the 'drumstick tree' (describing the shape of its pods), 'horseradish tree' (describing the taste of its roots), benzolive tree, kelor, marango, mlonge, moonga, mulangay, nébéday, saijhan, sajna or Ben oil tree is one of the best known and most widely distributed.

Tree morphology

This tree ranges in height from 5 to 12 m with an open, umbrella-shaped crown, straight trunk and corky, whitish bark, the tree produces a tuberous tap root. The evergreen or deciduous foliage (depending on climate) has leaflets 1 to 2 cm in diameter; the flowers are white or cream colored. The fruits (pods) are initially light green, slim and tender, eventually becoming dark green, firm and up to 120 cm long, depending on the variety. Fully mature, dried seeds are round or triangular, the kernel being surrounded by a lightly wooded shell with three papery wings. It was shown that the average relative masses of shells and kernel in *M.oleifera* were 27% and 73%, respectively.

Climate and soil conditions

The *M.oleifera* for its growth prefers hot, semiarid regions (annual rainfall 250-1500 mm), although it has been found to adapt well to hot, humid, wet conditions with annual rainfall in excess of 3000 mm.

Cultivation

It is already an important crop in India, Ethiopia, the Philippines and the Sudan, and is being grown in West, East and South Africa, tropical Asia and Latin America. India is the largest producer of *Moringa* with an annual production of 1.1 to 1.3 million tonnes of tender fruits from an area of 380 km^2 [Nepolean *et al.,* 2009].

Nutrition

M.oleifera is especially promising as a food source in the tropics. A large number of reports on the nutritional qualities of this plant now exist in both scientific and popular literature. Some organizations advocated *M.oleifera* as "*natural nutrition for the tropics*".

Leaves:

The leaves of *M.oleifera* and the residue obtained after the recovery of oil and coagulants can be good sources of proteins for animal feeds. These leaves can be eaten fresh, cooked, or stored as dried powder without loss of nutritional value. *Moringa* leaves contain more Vitamin A than carrots, more calcium than milk, more iron than spinach, more Vitamin C than oranges, and more potassium than bananas. The protein quality of *Moringa* leaves impersonate that of milk and eggs [Fahey, 2005].

Seeds:

Seeds of some *Moringa* varieties are consumed by humans after roasting and taste like groundnuts [Ramachandran *et al.*, 1980]. The sulphur-containing amino acids in kernel proteins are present at higher concentrations, but other essential amino acids are deficient. The high concentrations of ascorbic acid, oestrogenic substances and β-sitosterol, iron, calcium, phosphorus, copper, vitamins A, B and C, α-tocopherol, riboflavin, nicotinic acid, folic acid, pyridoxine, β-carotene, protein, and in particular essential amino acids such as methionine, cystine, tryptophan and lysine present in *Moringa* leaves and pods make it a virtually an ideal dietary supplement [Makkar and Becker, 1996].

Oil:

M.oleifera seed oil is pleasant tasting, highly edible and resembles olive oil in its fatty acid composition. In addition it possesses behenic acid (C 22:0), lignoceric acid (C 24:0) and traces of lauric *n*-pentadecanoic and pentadecenoid acids [Abdulkarim *et al.*, 2005].

Haemagglutination activity was also detected in the meal. Tannins, saponins, trypsin, amylase inhibitors, lectins, cyanogenic glucosides and glucosinolates were detected in twigs, leaves and stems but in low concentrations [Makkar and Becker, 1997].

Phytochemistry

M.oleifera is rich in compounds containing the simple sugar, rhamnose and a fairly unique group of compounds called glucosinolates and isothiocyanates [Fahey *et al.*, 2001]. Specific components of *Moringa* preparations that have been reported to have hypo-tensive, anticancer, and antibacterial activity include 4-(4'-*O*-acetyl-α-L-rhamnopyranosyloxy) benzyl isothiocyanate, 4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate, niazimicin, pterygospermin, benzyl isothiocyanate, and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate. While these compounds are relatively unique to the *Moringa* family, it is also rich in a number of vitamins and minerals as well as other more commonly recognized phytochemicals such as the carotenoids (including β-carotene or pro-vitamin A) [Bhattacharya *et al.*, 1982].

Flowers contain nine amino acids, sucrose, D-glucose, traces of alkaloids, wax, quercetin and kaempferat; the ash is rich in potassium and calcium. They have also been reported to contain some flavonoid pigments such as alkaloids, kaempherol, rhamnetin, isoquercitrin and kaempferitrin [Faizi *et al.*, 1994; Siddhuraju and Becker, 2003]. Anti-hypertensive compounds thiocarbamate and isothiocyanate glycosides have been isolated from the acetate phase of the ethanol extract of *Moringa* pods [Faizi *et al.*, 1998]. Cytokinins have been shown to be present in the fruit.

Lately, interest has been generated in isolating hormones/growth promoters from the leaves of *Moringa*. *Moringa* leaves act as a good source of natural antioxidant due to the presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics and carotenoids [Makkar and Becker, 1996; Anwar *et al.*, 2005]. The composition of the sterols of *Moringa* seed oil mainly consists of campesterol, stigmasterol, β-sitosterol and clerosterol [Tsaknis *et al.*, 1999].

Phytochemical analysis demonstrated the presence of the common phytoconstituents like tannins, phlobatannin, steroid, glycoside, saponin, glycoside, terpenoids and flavanoid in both the *Moringa oleifera* seed and flower extract. The presence of these constituents has been reported to account for the exertion of antimicrobial activity by plants [Nepolean *et al.*, 2009].

Medicinal uses and pharmacological properties

Moringa oleifera also has numerous medicinal uses, which have long been recognized in the Ayurvedic and Unani systems of medicine [Mughal *et al*., 1999]. The medicinal attributes are detailed in Table: 1.1. Other important medicinal properties of various parts of this plant are described below.

Leaves: *Moringa* leaf juice is known to have a stabilizing effect on blood pressure [Dahot, 1988]. The crude extract of *Moringa* leaves has a significant cholesterol lowering action [Ghasi *et al.*, 2000]. *Moringa* leaves are effective for the regulation of thyroid hormone status [Tahiliani and Kar, 2000]. A recent report showed that *M.oleifera* leaf may be applicable as a prophylactic or therapeutic anti-HSV (Herpes simplex virus type 1) medicine and may be effective against the acyclovir-resistant variant [Lipipun *et al.*, 2003]. The methanol fraction of *M.oleifera* leaf extract showed anti-ulcerogenic and hepatoprotective effects in rats [Pal *et al.*, 1995]. Makonnen *et al.*, [1997] found *Moringa* leaves to be a potential source for antitumor activity.

Seeds: *Moringa* fruit has been found to lower the serum cholesterol, phospholipids, triglycerides, low density lipoprotein (LDL), very low density lipoprotein (VLDL) [Mehta *et al.*, 2003]. With dual activity, antipollution and conditioning/strengthening of hair, the *M.oleifera* seed extract is a globally acceptable innovative solution for hair care [Anwar *et al*., 2007].

Root: *Moringa* roots have been reported to be rich in antimicrobial agents which are attributed by the presence of 4-α-L-rhamnosyloxy benzyl isothiocyanate and aglycone of deoxy-niazimicine (*N*-benzyl, *S*-ethyl thioformate). These are reported to contain an active antibiotic principle, pterygospermin, which has powerful antibacterial and fungicidal effects [Ruckmani *et al.*, 1998]. *M.oleifera* roots have been reported to possess antispasmodic activity [Caceres *et al.*, 1992].

Bark: The bark extract has been shown to possess antifungal activity, while the juice from the stem bark showed antibacterial effect against *Staphylococcus aureus* [Mehta *et al.*, 2003]. The flowers and leaves also are considered to be of high medicinal value with anti-helminth activity [Bhattacharya *et al.*, 1982].

Table: 1.1. Some common medicinal uses of different parts of *Moringa oleifera*

Plant part	Medicinal use	References
Root	Antilithic, rubefacient, vesicant, carminative, antifertility, anti-inflammatory, stimulant in paralytic afflictions; act as a cardiac/circulatory tonic, used as a laxative, abortifacient, treating rheumatism, inflammations, articular pains, lower back or kidney pain and constipation.	[The Wealth of India, 1962; Padmarao *et al.*, 1996; Dahot, 1988]
Leave	Purgative, applied as poultice to sores, rubbed on the temples for headaches, used for piles, fevers, sore throat, bronchitis, eye and ear infections, scurvy and catarrh; leaf juice is believed to control glucose levels, applied to reduce glandular swelling.	[The Wealth of India, 1962; Dahot, 1988;Morton, 1991; Makonnen *et al.*,1997]
Stem bark	Rubefacient, vesicant and used to cure eye diseases and for the treatment of delirious patients, prevent enlargement of the spleen and formation of tuberculous glands of the neck, to destroy tumors and to heal ulcers. The juice from the root bark is put into ears to relieve earaches and also placed in a tooth cavity as a pain killer, and has anti-tubercular activity.	[Bhatnagar *et al.*, 1961; Siddhuraju and Becker, 2003]
Gum	Used for dental caries, and is astringent and rubefacient; Gum, mixed with sesame oil, is used to relieve headaches, fevers, intestinal complaints, dysentery, asthma and sometimes used as an abortifacient, and to treat syphilis and rheumatism.	[Fuglie, 1999]
Flower	High medicinal value as a stimulant, aphrodisiac, abortifacient, cholagogue; used to cure inflammations, muscle diseases, hysteria, tumors, and enlargement of the spleen; lower the serum cholesterol, phospholipid, triglyceride, VLDL, LDL cholesterol to phospholipid ratio and atherogenic index; decrease lipid profile of liver, heart and aorta in hypercholesterolaemic rabbits and increased the excretion of faecal cholesterol.	[Bhattacharya *et al.*, 1982; Dahot, 1988; Siddhuraju and Becker, 2003; Mehta *et al.*, 2003]
Seed	Seed extract exerts its protective effect by decreasing liver lipid peroxides, antihypertensive compounds thiocarbamate and isothiocyanate glycosids have been isolated from the acetate phase of the ethanolic extract of *Moringa* pods.	[Faizi *et al.*, 1998; Lalas and Tsaknis, 2002]

Waste water treatment through coagulation

Water has been declared a human right by the United Nations. Inadequate sanitation and water services to the urban poor population are among the most serious challenges facing the developing world. According to UNESCO nearly one billion people lack access to safe drinking water. About 3.57 million people die each year from water-related disease. This can be dealt with water treatment plants. In developing countries treatment plants are expensive and inadequate treatment technology hamper in solving this problem. In water treatment process coagulation and flocculation is employed to separate suspended particles from the water. Coagulation is the destabilization of colloids by neutralizing the forces that keep them apart. Cationic coagulants provide positive electric charges to reduce the negative charge (zeta potential) of the colloids. As a result, the particles collide to form larger particles (flocks). Coagulation, thus, implies formation of smaller compact aggregates. Rapid mixing is required to disperse the coagulant throughout the liquid.

Commonly used chemicals for various water treatments are coagulants. These coagulants can be classified into inorganic coagulants, synthetic organic polymers and naturally occurring coagulant. These coagulants are used for various purposes depending on their chemical characteristics. An inorganic polymer "PAC" (polyaluminum chloride) and inorganic salt "alum" (aluminum sulfate) are the most widely used coagulants in water treatment [Boisvert *et al.,* 1997; Okuda *et al.,* 2001a]. Recently, synthetic organic polymers became widely used for water treatment [Robinson, 1979]. However, there is a fear that aluminum (the major component of PAC and alum) may induce Alzheimer's disease [Crapper *et al.,* 1973] and has strong carcinogenic properties [Mallevialle *et al.,* 1984]. They are also reported to associate with environmental problems [Christopher *et al.,* 1995].

On the other hand, naturally occurring coagulants are usually presumed safe for human health. Many natural coagulants from plants or animal origin can be effectively used for the treatment of drinking water [Kawamura, 1991; Ganjidoust *et al.,* 1997]. Some are used at household levels in traditional systems using crude (non-purified) extract. A number of effective coagulants have been identified from plant origin. Some of the common ones include *M.oleifera* [Jahn, 1988], nirmali [Tripathi *et al.,* 1976], okra [Al-Samawi and Shokrala, 1996], tannin from valonia [Özacar and Sengil, 2000], apricot, peach kernel, beans, maize and rice [Bhole, 1995; Jahn, 2001].

By using natural coagulants, considerable savings in chemicals and sludge handling cost may be achieved. Al-Samawi and Shokrala [1996] reported that 50-90% of alum requirement could be saved when okra was used as a primary coagulant or coagulant aid. Apart from being less expensive, natural coagulants produce readily biodegradable and less voluminous sludge.

Among all the naturally occurring coagulant removal sources *M.oleifera* (MO) seed is one of the most commonly known natural coagulants that can be used for water purification [Jahn, 1988]. Jahn [1981] first studied and confirmed the coagulating properties of *Moringa* seeds after observing women in Sudan use the seeds to clarify the turbid Nile waters. The use of *Moringa* species for water clarification is a part of African indigenous knowledge. Technically speaking the part that is used for water treatment is a waste product and it can be acquired at a very low cost. Ndabigengesere *et al.,* [1995] and Narasiah *et al.,* [2002] reported that sludge produced from *M.oleifera* coagulated turbid water was only 20-30% that of alum treated counterpart. However, some reports show *M.oleifera* coagulant has high coagulation activity only for high turbidity water this activity is low for low turbid water [Muyibi and Evison, 1995]. *Moringa oleifera* seed extract also has the ability to remove an anionic surfactant such as SDS from aqueous solutions while pH and temperature were found to be not very important factors in the removal efficiency [Heredia and Martin, 2008]. The exact mechanism of coagulation by *Moringa* is not well understood and different authors have attributed it to existence of proteins and non-protein flocculating agents [Ndabigengesere *et al.,* 1995; Gassenschmidt *et al.,* 1995; Okuda *et al.,* 2001b].

Moringa water treatment mechanism

The extract of *Moringa* seeds act as a coagulant which make suspended solids gather together into small flocs which later can be easily removed by filtration. *Moringa* seed powder contains positively charged small cationic protein which attracts the suspended water particles having negative electrical charge and forms floccules.

As a biosorbent for heavy metal removal

Heavy metal contaminants in the environment have become a concern due to the growing health risks to the public. Heavy metals are released into the aqueous environment through a variety of sources such as metal smelters, effluents from plastics, textiles, microelectronics, wood preservatives producing industries, usage of fertilizers and pesticides [Prasad and Hagemeyer, 1999]. Exposure to heavy metals can be carcinogenic and teratogenic, if not fatal [Carson *et al.,* 1986]. Because of the increasing environmental concern regarding heavy metal contamination, there has been a great deal of interest in the removal of heavy metal ions from contaminated soils and waste streams [Gardea-Torresdey *et al.,* 1998].

Methods traditionally employed for water remediation consist of removal of heavy metals by filtration, flocculation, activated charcoal, and ion-exchange resins. There has been a tremendous amount of attention given to the use of biological systems for the removal of heavy metal ions from contaminated areas [Cervantes and Gufierrez-Corona, 1994; Ramelow *et al.,* 1996].

Interest in using plants for environmental remediation is increasing due to their natural capacity to accumulate heavy metals, and degrade organic compounds [Nada Kumar *et al.,* 1995]. Biosorbents such as sunflower stalks [Sun and Shi, 1998], olive pomace [Pagnanelli *et al.,* 2003] and cocoa shells [Meunier *et al.,* 2003] have been explored for heavy metal removal. Some of the biosorbents from the plant sources are listed in Table 1.2. Since chemical functional groups are most likely responsible for the metal binding, it is likely that higher plant cells might also be capable of metal binding.

Crude extract (Biomass) of *Moringa* seeds could be used as a less expensive biosorbent for the removal of cadmium (Cd), Zinc (Zn) from aqueous media [Sharma *et al.,* 2006; Bhatti *et al.,* 2007]. The aqueous solution of *Moringa* seed is a heterogeneous complex mixture having various functional groups, mainly low molecular weight organic acids (amino acids). These amino acids have been found to constitute a physiologically active group of binding agents, working even at a low concentration, which because of the ability to interact with metal ions is likely to increase the sorption of metal ions [Brostlap and Schuurmans, 1988].

Table. 1.2. List of some bioadsorbents from the plant origin.

S.No	Adsorbent	Metal(s)	Reference
1	Maize bran	Pb(II)	[Singh *et al.*, 2006]
2	Rice husk, bran,	Cu(II), Cr(VI) and Pb(II)	[Wong *et al.*, 2003; Singh *et al.*, 2005a]
3	Peanut hull carbon	Hg(II), Cd(II), Pb(II), Ni(II), Cu(II)	[Periasamy and Namasivayam,1995;1996]
4	*Cassia fistula*	Ni(II)	[Hanif *et al.*, 2007]
5	*Azadirachta indica* (Neem) leaf powder	Pb(II)	[Bhattacharyya and Sharma, 2004]
6	*Medicago sativa*	Cd(II), Cr(III,IV), Pb(II), Zn(II)	[Gardea-Torresdey *et al.*, 1998]
7	*Datura innoxia*	Cd(II)	[Delhaize *et al.*, 1989]
8	*Quercus ilex* L.	Cr(II), Ni(II),Cd(II), Pb(II)	[Prasad and Freitas, 2000]
9	*Prosopis juliflora* DC.	Pb(II),Cu(II), Cd(II)	[Jayaram and Prasad, 2009; Senthilkumar *et al.*, 2005]
10	*Caladium bicolor* (Wild Cocoyam)	Pb(II), Cd(II)	[Jnr and Spiff, 2005]
11	*Strychnos potatorum*	Pb(II)	[Jayaram *et al.*, 2009]
12	Waste banana pith	Acid violet, Congo red, Rhodamine	[Namasivayam and Kanchana, 1992]
13	Peanut hull	Hg(II)	[Namasivayam and Periasamy, 1993]
14	Water hyacinth roots	Basic dyes(Methylene blue and Victoria blue)	[Low *et al.*, 1995]
15	Husk of Bengal gram (*Cicer arientinum*)	Cr(VI)	[Ahalya *et al.*, 2005]

Some biologically active proteins in the seeds (With special reference to lectins and glycosidases)

Plant seeds contain several biologically-active proteins that play various specialized functions. The most representative molecules are hydrolytic enzymes [Henrissat *et al.,* 2001], lectins [Rüdiger and Gabius, 2001], ribosome inactivating proteins [Parkash *et al.,* 2002], lipid transfer proteins [Cammue *et al.,* 1995], thaumatin-like proteins [Wurms *et al.,* 1999], protease inhibitors [Joshi *et al.,* 1998], cyclophilin-like proteins [Ye and Ng, 2000], chitinases [Gozia *et al.,* 1995], peroxidases [Ye and Ng, 2002], defensins [Wong and Ng, 2005] and novel proteins and peptides [Terras *et al.,* 1993; Lee *et al.,* 1995]. Some of these seem to be essential for the plant defense process, while others have physiological role in plant development.

Lectin

Definition: Definition of a Lectin (From the Latin verb *legere* (meaning "to select") (proposed by Boyd and Shapley in 1954)

According to Peumans and Van Damme, Lectins are defined as a class of proteins which possess at least one non-catalytic domain capable of specific recognition and reversible binding to carbohydrate (glycan) [Peumans and Van Damme, 1995]. To qualify as lectin today, a (glyco) protein must meet three distinct requirements [Goldstein *et al.,* 1980; Rüdiger and Gabius, 2001]

- A lectin is a (glyco) protein that binds carbohydrate.
- Lectins are distinct from immunoglobulins.
- Lectins do not biochemically modify the carbohydrates which they bind.

They are ubiquitously distributed in nature and most abundant in the Plant kingdom, where they can be found in seeds, leaves, barks, bulbs, rhizomes, roots and tubers depending on the plant species [Hankins *et al.,* 1988; Diaz *et al.,* 1990; Zhu *et al.,* 1996; Wright *et al.,* 1999]. Table: 1.3. Describes the historical aspects of plant lectins.

Table: 1.3. Historical back ground of plant lectins.

Date	Investigators	Discovery
1860	S. Weir Mitchell	Lectin like activity form venom of the rattlesnake.
1888	H. Stillmark	*Ricinus communis* plant extract has hemagglutinating properties.
1890	P. Ehrlich	Lectins (ricin and abrin) used as immunogens in early immunological studies.
1908	K. Lansteiner and H. Raubitsheck	Different hemagglutinating properties found in extracts of various plant seeds.
1919	J. Sumner	Crystallization of Con A.
1936	J. Sumner	Lectins bind sugar - Con A precipitates glycogen.
1940	W. Boyd, R. Reguera and K.O. Renkonen	Lectins specific for some human blood group antigens.
1952	W. Watkins and W. Morgan	Use of lectins and glycosidases to prove that blood group antigens are sugars and to deduce the structures of the antigens.
1954	W. Boyd and E. Shyleigh	The name lectin is proposed to replace hemagglutinin.
1960	P.C. Nowell and J.C. Aub	Red kidney bean lectin *P.vulgaris* mitogenic for resting lymphocytes.
1960's &1970's	M. Burger and G. Nicolson	Lectins preferentially agglutinate some animal tumor cells.
1980's	Kornfeld(s) Osawa, and Kobata	Use of immobilized lectins to analyze animal glycoconjugates.
1980's	D. Kabelitz and D.J. Gee,	Discovery that plant lectins induce apoptosis.

Occurrence of lectins in plants

Lectins are widely found in nature [Vandamme *et al*., 1998; Rüdiger *et al*., 2000] in most organisms such as the plants, animals, viruses, bacteria and fungi. The richest source for most lectins is the seeds or, more generally, the storage organs of plants. Within the cells, lectins are primarily found in protein bodies. They are synthesized in the endoplasmic reticulum (ER) and transported via the Golgi apparatus and originate by subdividing the vacuole [Rüdiger and Gabius, 2001]. Viewed from their origin and their role for protein turnover, protein bodies in seeds are related to lysosomes in eukaryotes. The main content of protein bodies are storage proteins lectins, hydrolases (glycosidases, phosphatases) and phytin used to store phosphate [Münz, 1989; Vitale and Chrispeels, 1992].

Classification of plant lectins

Lectins represent a heterogeneous group of proteins with wide diversity in size, structure, physicochemical properties, biological activities, sugars specificities, composition and architecture of binding sites. The majority of plant lectins can be classified in seven families of structurally and evolutionary related proteins [Van Damme *et al*., 1998]. This classification is dealt with briefly below:

1. Amaranthin lectins
2. Chitin binding lectins [Hevein-like]
3. Cucurbitaceae phloem lectins
4. Monocot mannose binding lectins
5. Type 2 RIP
6. Legume lectins
7. Jacalin-related lectins

Lectins that do not fall into any of the above families are referred to as unclassified lectins.

Biological functions of plant lectins

Despite the enormous interest in plant lectins, little is known about their functions in the plant. Lectins probably have many different and important functions in plants. Some of these functions are described briefly here.

Symbiosis: Lectins play a role in symbiosis by recognition of some elements in nitrogen-fixing bacteria (Rhizobia). There is a high level of research interest in the possibility that leguminous lectins may be involved in rhizobial attachment and root nodulation. Some of the nodulation factors released by the bacteria are glycoconjugates, and it is intriguing to consider that some of the members of the plant lectin group may be involved in this important plant signaling pathway.

Protection from microorganisms and animal predators: Lectins are thought of acting as plant antibodies. The carbohydrate binding properties of the lectins make them useful probes in distinguishing the microorganism. Suggestive evidence for their defense role had come from their interactions with the fungi. Investigators explored the role of lectins in plant defense against seed predators (insects or fungi) or during seedling development [Etzler, 1998; Naeem, 2001].

Lectins have thus been proposed to protect plants during imbibition, germination and early growth of seedlings [Mirleman *et al*., 1975; Barkai Golan *et al*., 1978]. Seed lectins may help to protect cotyledons from bacteria that degrade the seed coats [Jones *et al*., 1964]. Due to their interaction with the digestive tract, the lectins from *Phaseolus vulgaris* (PHA) are toxic for mammals in general [Liener, 1986].

Protection from insects; transgenic plants: Plant lectins are toxic to many plant pathogens, and this may be a major role within seeds and other peripheral tissues of the plants. For example, the snowdrop lectin *Galanthus nivalis* agglutinin is toxic towards the sap-sucking insect called the rice brown plant hopper. Transgenic expression of the gene encoding *G.nivalis* agglutinin in rice plants decreases survival and fecundity of insects attacking the transgenic plants.

Interaction with enzymes: The interaction of lectins with storage proteins is clearly only one aspect of the interactions of lectins within the protein body. In addition to storage proteins, also hydrolytic enzymes (glycosidases) [Einhoff *et al.*, 1986; Freier and Rüdiger, 1987] and phosphatases [Rüdiger and Bartz, 1993] are bound by the lectin in a carbohydrate-, ion strength- or pH-dependent manner. A striking example is the α-mannosidase from *Canavalia ensiformis* seeds which despite being a glycoprotein reacts with the lectin from the same plant, ConA, not via its carbohydrate moiety but by ionic interaction most effectively at pH 5 [Einhoff and Rüdiger, 1986a; Einhoff and Rüdiger, 1986b].

The dual binding ability of lectins to storage proteins and to protein body membranes suggests that lectins might form reversible glue between protein and membrane, an idea that is supported by the time course of lectin and storage protein biosynthesis during seed maturation [Wenzel *et al.,* 1993]. Lectins may not only bind enzymes but also modify their activities. In addition, some lectins are closely associated with an α-galactosidase activity [Brechtel *et al.,* 2001]. Hankins and Shannon [1978] isolated a hemagglutinin from mung bean seeds that had strong α-galactosidase activity.

Other cell recognition functions: The interaction of the pollen and the pistil is thought to be due to the interaction of the glycoproteins on the cell surface of the stigma or the style. This interaction leads to either the retardation of growth or penetration of the incompatible pollen tubes or the promotion of the growth of the compatible pollen tube through the style to the ovary [Heslop-Harrison, 1978]. Example, Con A was found to react specifically with receptors on the stigma surface and to block the penetration of the stigma cuticle by the pollen tube [Knox *et al*., 1976].

Cell wall elongation: The finding of the lectin non-covalently associated with the cell walls of mung bean seedlings led Kauss and Glaser [1974] to propose that lectins may serve as a non-covalent 'glueing' substance in the cell wall and play a role in cell wall extension by participating in the breaking and reformation of non-covalent cross links with other wall components.

Applications of plant lectins

Although the natural functions of plant lectins are just now being understood, investigators over the years have found many uses for plant lectins. Table: 1.4 gives some details on the applications of plant lectins.

Table: 1.4. Some uses for plant lectins as tools in basic and medical sciences

Field	Application
Biochemistry	Detection of defined carbohydrate epitopes of glycoconjugates in blots or on thin-layer chromatography plates
	Purification of lectin-reactive glycoconjugates by affinity chromatography
	Glycan characterization by serial lectin affinity chromatography Glycome analysis (glycomics)
	Quantification of lectin-reactive glycoconjugates in enzyme-linked lectin-binding assays (ELLA)
	Quantification of activities of glycosyltransferases/glycosidases by lectin-based detection of products of enzymatic reaction.
Cell biology	Characterization of cell surface presentation of glycoconjugates and their preceding intracellular assembly and routing in normal and genetically engineered cells
	Analysis of mechanisms involved in correct glycosylation by lectin-resistant cell variants
	Fractionation of cell populations
	Modulation of proliferation and activation status of cells
Medicine	Detection of disease-related alterations of glycan synthesis
	Blood group typing and definition of secretor status
	Quantification of aberrations of cell surface glycan presentation.
	Cell marker for diagnostic purposes incl. infectious agents (viruses, bacteria, fungi, parasites)

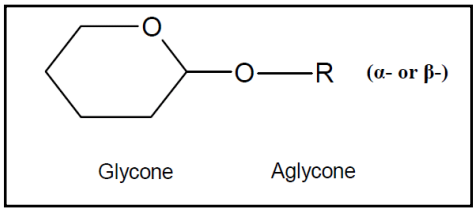
Glycosidases (PLANT HYDROLASES)

Carbohydrates

Carbohydrates constitute the major class of organic compounds in the plant tissues. During plant life, the carbohydrates play an important role in various physiological functions such as growth, source of carbon and energy, defense against pathogen attack, signaling, mechanical resistance, and interactions with the environment [Zagrobelnyet *et al.*, 2004; Thompson, 2005; Lloyd *et al.*, 2005; Parre and Geitmann, 2005; Wingler *et al.*,2006]. Carbohydrates are found in cell wall polysaccharides, storage material, glycolipids, glycoproteins, glycosides and in various other non-carbohydrate moieties such as steroids, hormones and lignin precursors [Henrissat *et al.*, 2001].

Glycosides

Formally, glycosides are compounds in which a sugar group is bonded through its anomeric carbon to another group *via* a glycosidic bond. In addition, sugar bonded to a non-sugar by an acetal linkage at carbon atom 1 to a noncarbohydrate residue for the molecule to qualify as a glycoside, thus excluding polysaccharides. The sugar group is then known as the glycone and the non-sugar group as the aglycone (alcohol, glycerol, phenol etc.).



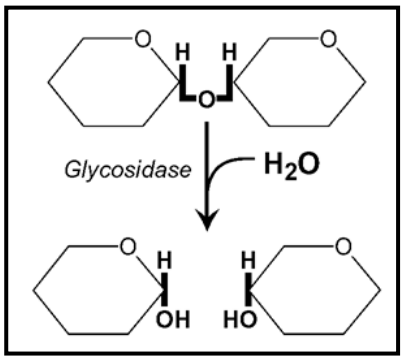
The sugar moiety can be joined to the aglycone in various ways:

1. Oxygen *(O-glycoside)*
2. Sulphur *(S-glycoside)*
3. Nitrogen *(N-glycoside)*
4. Carbon (*C-glycoside)*
5. *α-Glycosides* and *β-glycosides* are distinguished by the configuration of the hemiacetal hydroxyl group.

Functions: Glycosides play numerous important roles in living organisms. Many plants store chemicals in the form of inactive glycosides. The structural and functional diversity of carbohydrates implies a vast spectrum of enzymes involved in their synthesis (glycosyltransferases), modification (carbohydrate esterases) and breakdown (glycoside hydrolases and polysaccharide lyases).

Glycoside hydrolases (GHs) (also called **glycosidases**)

According to CAZy database (http://www.cazy.org) Glycoside hydrolases are a widespread group of enzymes which hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Glycosyl hydrolases transfer the glycosyl bond to a water molecule; glycosyl transferases transfer this glycosyl bond to the -OH group of another glycosyl residue. Enzymes, which catalyze the hydrolysis of glycosidic linkages are widely distributed in nature and include α-galactosidase, α-mannosidase, β-galactosidase, invertase, maltase, β-glucosidase, amylase etc. Glycosidases are also known as carbohydrolases. The existence of glycosidases has been known for more than 100 years and they were the very first biological catalysts investigated.



Occurrence and importance:

Glycoside hydrolases are found in essentially all domains of life. Plant GH enzymes have been reported to be located in different compartments of cells such as the cytoplasm, cell wall, membrane, vacuole, endoplasmatic reticulum (ER) and peroxisomes [Roitsch and Gonzalez, 2004; Minic and Jouanin, 2006]. The physiological and biochemical function of only a small proportion of the identified GHs have been experimentally demonstrated. These enzymes are supposed to play various functions in cell wall metabolism, plant defense, signaling and hydrolysis of starch [Henrissat *et al.,* 2001].

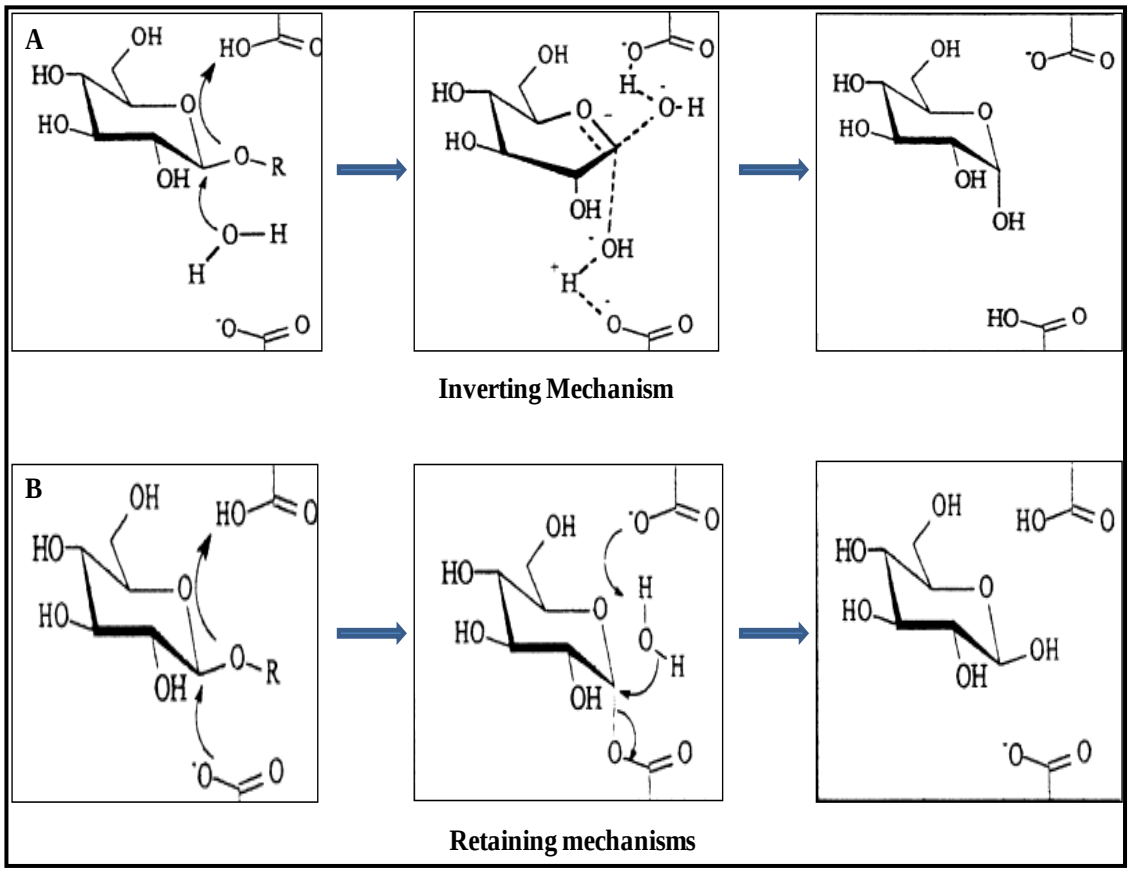
Some of the known functions of plant GHs

- GHs -- in metabolism of cell wall polysaccharides
  - Biosynthesis of cell wall polysaccharides
  - Assembly and reorganization of the cell wall
- Biosynthesis and remodulation of glycans
- Mobilization of storage reserves
- The plant defense
- Symbiosis
- Secondary plant metabolism
- Signaling
- Glycolipid metabolism

Mechanism of the glycosyl hydrolases

Enzymatic hydrolysis of glycosidic bonds is carried out with two major mechanisms giving rise to either an overall retention, or an inversion, of anomeric configuration [Koshland, 1953; Sinnott, 1990; McCarter and Withers, 1994]. In the proposed mechanism for inverting enzymes the glycosidic oxygen is initially protonated by a general acid catalyst, followed by a nucleophilic attack of a water molecule on the C_1 atom of the sugar, which is activated by a carboxylate base [Figure: 1A], leading to inversion of the anomeric conformation. This mechanism is known as a single displacement mechanism [Koshland, 1953], bond breaking and bond making both proceed in a single concerted step. The reaction rate depends on the concentrations of both nucleophile and substrate. The retaining reactions proceed via a double-displacement mechanism [Vernon, 1967], the first step involves a similar protonation of the glycosidic oxygen by a general acid as in the inverting mechanism, creating an intermediate, which is attacked by a water nucleophile assisted by the base form of the acid catalyst in a second step [Figure: 1B]. Each step inverts the configuration of the anomeric carbon. The two displacement steps therefore create an overall retention of the configuration. For retaining enzymes the intermediate could either be an oxo-carbonium ion which is electrostatically stabilized by a carboxylate, or involves formation of a covalent bond, in which one of the catalytic aspartates (in some cases a glutamate) is presumed to act as a nucleophile.

Figure: 1.1. Catalytic mechanism of glycosidases; (**A**) Inverting mechanism (**B**) Retaining mechanism



Classification of Glycosyl Hydrolases

Glycoside hydrolases can be classified in many different ways.

Endo/exo

Glycosidases are classified into exo-glycosidases and endo-glycosidases based on the nature of hydrolysis. The exo-glycosidases (e.g. galactosidases, glucosidases) act on the glycosidic bond present at the non-reducing end of the saccharide chain whereas endo-glycosidases (e.g. amylases) act on the glycosidic bond within the saccharide chain.

Enzyme Commission (EC) number

The IUB Enzyme Nomenclature (*i.e.*, EC number) is based upon the substrate specificity. Glycosylhydrolases are denoted as EC 3.2.1.x, where the first three numbers indicate that the enzyme hydrolyzes and the last position specifies the substrate.

Mechanistic classification

Two reaction mechanisms are most commonly found for the retaining and inverting enzymes, as first outlined by Koshland as exemplified above.

Sequence-based classification

The current, more popular, classification method was proposed by Henrissat in 1991 and is based upon primary sequence similarities as determined by hydrophobic cluster analysis. Glycoside hydrolases from various sources, including bacteria, fungi, plants and animals were classified into 125 families based on alignment of amino acid sequences (as on June 2011). The compilation is regularly updated on the CAZY website (http://www.cazy.org/CAZY/) [Henrissat, 1991, 1998; Henrissat *et al.*, 2001]. At present these glycoside hydrolase families are divided into 14 clans (GH A- GH N).

These enzymes were characterized using biochemical, molecular and genetic approaches. The physiological and biochemical function of only a small proportion of the identified GHs have been experimentally demonstrated (http://afmb.cnrs-mrs.fr/CAZY).

In this thesis we focused on two glycosidases (α-mannosidase and α-galactosidase) from *Moringa oleifera* seed kernel which are found to be in high concentration compared to other glycosidases detected.

Mannosidase

It is an enzyme which can remove mannose residues from the glycosidic linkage of the glycoproteins and oligosaccharides.

There are two types of mannosidase:

- α-mannosidase
- β-mannosidase

α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) is an enzyme that catalyzes the hydrolysis of terminal, non-reducing α-D-mannose residues in α-D-mannosides, storage glycoproteins and can cleave all known types of α-mannosidic linkages. They mainly cleave α-(1-2), α-(1-3) and α-(1-6) linked.

β-mannosidase (β-D-mannoside mannohydrolase, EC 3.2.1.25) is an enzyme which mainly cleaves β-(1-4)-linked glycoproteins and oligosaccharides.

Occurrence, sources and significance

α-mannosidase is widely distributed in animals, plants and microorganisms. Among plants they are isolated from different plant tissues. Thought to be accumulated more in the seeds it has also been isolated from different parts like fruit of watermelon [Nakagawa *et al.,* 1988], *Lycopersicon esculentum* II [Suvarnalatha and Prabha, 1999; Hossain *et al.,* 2009], *Capsicum annuum* [Priya Sethu and Prabha, 1997], kiwifruit [Ogawa *et al.,* 1990] and grape [Burns and Baldwin, 1994]. Other sources of α-mannosidase include lyophilized latex of babaco (*Vasconcellea heilbornii*) [Blom *et al.,* 2008], cotyledons of *Lupinus angustifolius* [Plant and Moore, 1982], *Phaseolus vulgaris* [Van der Wilden and Chrispeels, 1983], hypocotyls of *Helianthus annuus* L [Lopez-Valbuena, *et al.,* 1989], leaves of *Canavalia ensiformis* [Niyogi and Singh, 1988] isoenzymes of barley leaves [Betz *et al.,* 1992], aleurone layers of resting wheat grains [Conti *et al.,* 1987], coleoptiles of *Avena sativa* [Greve and Ordin, 1977]. Table: 1.5. Summarizes some of the known α-mannosidases from plant seeds.

Table: 1.5. Molecular properties of α-mannosidase from some plant seeds.

S.No	plant	MW: Native; Subunit	pH	°C	Gp%	Metal	Reference
1	*Canavalia ensiformis*	230kDa[a];66kDa,44kDa[b]	5.0	50°C	---	Zn^{2+}	Snaith, 1975
2	Indian lab lab beans	195kDa±5kDa[a];66kDa,44kDa[b]	---	40°C; 20 min	4.5%	---	Tulasi and Nadimpalli, 1997.
3	Triticale	195kDa±5kDa[a];58kDa,40kDa[b]	---	50°C; 20 min	7.0%	---	Mahadevi *et al*, 2002
4	*Carica papaya*	156kDa[a]; 78kDa,78kDa[b]	5.5-10.0pH	>60°C	---	Zn^{2+}	Ohtani and Misaki, 1983
5	*Medicago sativa* L	220kDa[a]; 75kDa,60kDa,50kDa,45kDa[b]	---	---	---	---	Curdel and Petek, 1980;Prijcker *et al.*, 1974
6	*Phaseolus vulgaris*	I 210kDa[a]; 2(110kDa)[b]: II 220kDa[a]; 2(110kDa)[b]	---	---	8.3%: 16.5%	Zn^{2+}	Paus, 1977
7	*Oryza sativa L*	190kDa[a]; 54kDa,42kDa[b]	4.3-4.5	---	---	Zn^{2+}	Kishimoto *et al.*, 2001
8	*Ginkgo biloba*	340kDa[a],120kDa[b]	5.0	---	---	Co^{2+}	Woo *et al.*, 2004
9	*Erythrina indica*	124kDa[a],127kDa[b]	4.6	50°C	8.6%	Zn^{2+}	Kestwal and Bhide, 2005
10	*Artocarpus communis*	I 75kDa[b]: II 60kDa[b]	5.6	60°C	---	Zn^{2+};Ca^{2+}	Ahi *et al.*, 2007
11	*Prunus serotina*	150kDa[a]	---	---	---	---	Waln and Poulton, 1987
12	*Almond*	250kDa[a]; 69,55,38,14kDa[b]	3.7				Misaki *et al.*, 2003

[a] Molecular Weight (MW) determined by gel filtration. [b] MW determined by SDS-PAGE.

pH: Optimal pH. °C: Optimal temperature & stability. Gp%: Glycoprotein %.Metal: Metal ion required.

Significance of α-mannosidase in plants

Plant *N*-linked glycoproteins are highly abundant and most of the proteins of the vacuole and the apoplast are modified by *N*-glycans (the vacuole of a typical mature plant cell occupies approximately 90% of the total cell volume). The search for the function of *N*-glycosylation of plant proteins has indicated that the *N*-linked glycans are essential. In general, protein glycosylation seems to be vital for the stability of secreted plant proteins. *N*-glycans also seem to have a role in the activation of proteins. In addition, *N*-linked glycoproteins appear to be essential for important cellular processes such as the maintenance of the cell cycle especially in developing or growing cells. In contrast to these general findings, the study of individual plant glycoproteins has revealed very little of the functions of specific *N*-glycans bound to specific glycosylation sites. The acid hydrolase α-mannosidase, which accumulates in plant vacuoles and probably is involved in the catabolism and turnover of *N*-linked glycoproteins.

The apparently universal occurrence of α-mannosidase in mammals and higher plants, as opposed to the virtual absence of β-mannosidase, suggests that, in many glycoproteins at least, the mannose residue is α-linked [Snaith and Levvy, 1968]. α-mannosidases (EC 3.2.1.24) are implicated in signal transduction by way of deglycosylation in crucial biochemical events both in animals and microbial systems [Jagadeesh *et al.,* 2004]. α-mannosidase also accelerates the glycoprotein and glycolipid degradation [Hosokawa *et al.,* 2001]. The deficiency of mannosidase in animals causes mannosidosis leading to the accumulation of undegraded glycoproteins having α-mannoside residues in their oligosaccharide part [Sun *et al.,* 1999]. Increased activity of α-mannosidase during fruit ripening has been reported for tomato, grape, muskmelon, olive, pear and watermelon [Suvarnalatha and Prabha, 1999]. Though, α-mannosidase has a role in germination and ripening the exact mechanism is not yet fully understood.

Exoglycosidases are useful tools for the structural analysis of glycoproteins. If an enzyme is to be used for this purpose, it has to be in a highly homogenous form.

Classification of α-mannosidase

α-mannosidases have been classified into two independently derived groups, Class I and Class II, based on their biochemical properties, substrate specificity, inhibitor profiles, catalytic mechanism and characteristic regions of conserved amino acid sequences and sequence alignments [Daniel *et al.,* 1994; Moremen *et al.,*1994; Eades *et al.,* 1998].

Class I: Class I includes α1-2, linkage specific mannosidase I, localized in the Golgi. They are inhibited by pyranose analogs such as deoxymannojirimycin (DMJ) and Kifunensine (Kf). In the glycosyl hydrolase (GH) classification, they are assigned to the inverting family 47. Class I α-mannosidase can further be divided into three subgroups, based on their activity toward $Man_9GlcNAc_2$ [Herscovics, 2001].

Class II: Class II includes α1-3 and α1-6, linkage specific mannosidase II, they are more heterogeneous group of the lysosomal mannosidases. They are inhibited by furonase analogs such as swainsonine. These enzymes are mainly involved in glycan catabolism. Class II mannosidase belong to the retaining GH family 38. Table 1.6 gives details of some of the properties of class I and class II mannosidases.

Applications

The mannosidases have found application in the food and pharmaceutical industry for production of fruit juices, degradation of plant material [Christgau *et al.,* 1994], coffee extraction [McCleary, 1990] and for the synthesis of oligosaccharides [Akino *et al.,* 1988]. Mannosidases are applied in combination with xylanases in the pulp and paper industry to partially breakdown mannan and xylan in softwood pulps. This leads to a significant reduction in the amount of chemical required for bleaching [Viikari *et al.,* 1994; Lahtinen *et al.,* 1995]. α-mannosidase has been used as a probe in the structural elucidation and functional studies of biologically important glycoproteins and glycolipids containing α-linked D-mannose. The enzyme has been used for the structural elucidation of glycoproteins and glycolipids. In particular, the α-mannosidase from jack bean has generally been employed in determining the glycan structure because its enzyme properties such as optimum pH, kinetics and substrate specificity have been extensively characterized.

Table: 1.6. Comparison of α-mannosidase class I and class II

Comparison of α-mannosidases	**Class- I**	**Class-II**
Glycosyl hydrolase classification	family 47(ER)	family 38(Golgi)
Metal ion dependence	Ca^{2+}	Zn^{2+}
Mannose linkage hydrolyzed	α 1,2	α1,2; α1,3 ; α1,6
Stereochemistry of product	inverted	retained
Optimal inhibitors	pyranose analogues	furanose analogues

Galactosidases

It is an enzyme which can remove galactose residues from the glycosidic linkage of the glycoproteins and oligosaccharides.

There are two types of galactosidases:

- β-galactosidase
- α-galactosidase

β-galactosidases

β-galactosidases (β-D-galactoside galactohydrolase or lactase; EC 3.2.1.23) catalyze the hydrolysis of the β-D-galactosyl bond. On the basis of amino acid similarities, β-galactosidases have been divided into four glycoside hydrolase (GH) families: 1, 2, 35 and 42. Most of β-galactosidases belong to family 2, whereas those from thermophilic, psychrophilic and halophilic organisms belong to GH family 42.

α-galactosidases

α-galactosidase (α-D-galactoside galactohydrolase E.C.3.2.1.22) is an exoglycosidase that catalyzes the hydrolysis of α-D-galactosidic linkages from a wide range of substrates including oligo-saccharides of raffinose family sugars; raffinose, stachyose, melibiose, verbascose and polysaccharides of galactomannans; locust bean gum and guar gum. More over it also hydrolyzes glycoconjugates; glycoproteins and glycosphingolipids. [Systematic name: α-D-galactoside galactohydrolase.]

Glycoside hydrolase families, which include α-galactosidases

GH4-α-galactosidase from *Escherichia coli.*

GH27-mainly α-galactosidases from Eukaryota (animals, plants, fungi, etc.) and some Eubacteria.

GH36-mainly α-galactosidases from Eubacteria and Eukaryota (fungi and plants).

GH57-α-galactosidases from *Pyrococcus furiosus* and *Thermococcus alcaliphilus.*

GH110-α-galactosidase from *Bacteroides fragilis.*

Occurrence, sources and significance

α-galactosidases are widely distributed in nature among plants, animals and microorganisms. This enzyme is first time reported from the sweet almond emulsion [Helferich *et al.,* 1932]. α-galactosidase activities associated with various physiological processes have been observed in different plant tissues, like leaves of *cucurbita pepo* and Runner bean [Sastry and Kates, 1964; Thomas and Webb, 1977], Roots of Maize(corn) [Hadacova and Benes, 1977], stalks of *Saccharum officinarum* (sugar cane) [Chinen *et al.,* 1981], endosperm of coconut [Balasubramaniam and Mathew, 1986] and *Euphorbia heterophylla* L [Suda *et al.,*2003] tomato fruit, melon fruit [Sozzi *et al.,* 1996; Gao and Schaffer, 1999; Feurtado *et al.,* 2001], grape flesh [Kang and Lee, 2001] cultured rice [Kim *et al.,* 2002], tubers of *Stachys Affinis* [Kato *et al.,* 1982], germinating coffee beans [Shen *et al.,* 2008] and in the cereal of barley [Chrost *et al.,* 2004]. It is also cloned and expressed in rice (*Oryza sativa*) [Chien *et al.,* 2008; Li *et al.,* 2007], *Cyamopsis tetragonoloba* (guar) [Overbeeke *et al.,* 1989], *Lycopersicon esculentum* Mill [Feurtado *et al.,* 2001]. Table: 1.7. Summarizes the general properties of the α-galactosidase isolated and studied from different seeds.

Significance

In plants, α-galactosidase is believed to be involved in a variety of processes, most importantly in the hydrolysis of oligo-saccharides such as raffinose and stachyose during the early germinative period, resulting in the liberation of free sugars, which may serve as a ready energy source for the growing plant [Dey and Pridham, 1972]. The role of α-galactosidase and β-mannanase in the hydrolysis of cell wall storage polysaccharides such as galactomannans and mannan at the latter period of germination of seed has been established [McCleary, 1975]. α-galactosidase is an important enzyme in coconut and plays role during germination and cell wall development [Balasubramaniam and Mathew, 1986]. The enzyme may have a synthetic function, as transgalactosylation reactions have been reported for α-galactosidases [Dey, 1979]. Its role has been implicated in the removal of toxic accumulants and to hydrolyze phenolic glycosides which could provide a means of control of the levels of plant growth substances [Strobel, 1974].

Table: 1.7. Molecular properties of α-galactosidase from some plant seeds.

Plant (Scientific Name)	Molecular weight (kDa)	pH optimum	Temperature optimum	Specificity	Reference
Coffea canephora, Coffea arabica	34kDa[a],36.7kDa[b]; 38.8kD[b]	6.3	55°C	---	Haibach *et al.*, 1991; Shen *et al.*, 2009
Artocarpus integriflia	35kDa[a]; 9.5kDa[b] x 4	5.2	---	melibiose	Appukuttan and Basu, 1987
***Glycine max* sps.**	160kDa[a]; 38kDa[b],40kDa[b]	---	---	raffinose, stachyose	Guimarães *et al.*, 2001
Lens culinaris	I160kDa[a]: II 40kDa[a]	6.1: 4.7	---	raffinose	Dey et al., 1983
Trifolium repens	41-43kDa[b]	3.8-4.2	---	---	Williams *et al.*, 1977
Prunus dulcis	33kDa[a]	5.5-5.7		melibiose	Malhotra and Dey, 1967
Helianthus Annuus	40kDa[a]; 41kDa[b]	5.0	50°C	raffinose	Kim *et al.*, 2003
Arachis hypogaea	30kDa b	6.0	50°C	raffinose	Bryant and Rao, 2001
Vigna unguiculata	33kDa[a]	6.0	50°C	---	De Oliveira-Neto *et al.*, 1998
Solanum lycopersicum	34kDa[b]	4.0	37°C	---	Calci *et al.*, 2009
***Tachigali multijuga* Benth**	34kDa[a]; 38kDa[b];	5.0-5.5	50°C	melibiose	Fialho *et al.*, 2008

[a] Molecular Weight (MW) determined by gel filtration. [b] Molecular Weight (MW) determined by SDS-PAGE.

Biotechnological applications of α-galactosidases

α-galactosidase has many potential applications in biotechnology and medicine [Dey, 1979; Ganter *et al.*, 1988; Somiari and Balogh, 1995].

a) Processing of legume based foods

Legumes thought to constitute an important diet throughout the world contain antinutritional factors such as galacto-oligosaccharides of raffinose family. Enzymatic treatment with microbial α-galactosidase offers promising solution for elimination of these oligo-saccharides, especially in soymilk.

b) Processing of sugar beet molasses

Raffinose elimination by the action of α-galactosidase in beet syrups facilitates crystallization and consequently improves the yield of the sucrose [Suzuki *et al.*, 1969].

c) Guar gum processing

Galactomannans have many industrial applications in pharmaceuticals, cosmetics, paper products, paints and plastics, well drilling and mining and explosives. Many investigators have developed processes for the modification of galactomannan polymers by using plant and microbial α-galactosidases.

d) ***Animal feed processing***

Many attempts were made to add exogenous α-galactosidase enzyme to the animal feed to increase the digestive efficiency and to improve the nutritive value.

e) Clinical significance and applications

At present time there is increased interest of α-galactosidase in human medicine. It plays crucial role in treatment of Fabry's disease, xenotransplantation and in blood group transformation.

f) Other potential applications of α-galactosidases

Transgalactosylation activity of α-galactosidases has frequently been used for the synthesis of new saccharides. In paper and pulp industry α-galactosidases could enhance the bleaching effect of β-1, 4 mannanase on soft wood Kraft pulp.

Scope of present investigation

M.oleifera belongs to the family *Moringaceae*. A single genus with 14 known species, *M.oleifera* is the most widely known and utilized of these [Morton, 1991]. Almost every part of this multipurpose tree is of good value not only as a food but also as medicine. It is one of the good examples of widely exploited species in the plant kingdom. To date, several studies have been focused on its nutritional aspects and medicinal uses of the *M.oleifera* tree parts. The pods, leaves and flowers are important sources of food. The leaves are specially, rich in vitamins, minerals and proteins. The roots and other parts of the tree are used in traditional medicine. Oil can be extracted from the seed and used in food preparation, fine lubrication of delicate machines and in the cosmetics industry. Further, many reports highlight the importance of the phytochemical constituent of this plant which plays a significant role in many biological processes. Due to these reasons it is often referred as a "miracle tree" by some to the researchers. The use of *M.oleifera* for waste-water treatment at household level is common in some areas of Africa and Asia [Jahn, 1988]. Recently efforts are being made to use it for water purification at treatment plants for community water supply. However, little or no studies have been done on the biochemical and biophysical properties of the component responsible for these properties. Furthermore, the water clarifying properties of this plant were studied using total seed powder. Although preliminary studies are under way in different laboratories to use the *M.oleifera* seed, as water clarifying agent no reports are available to date on the usage of the isolated seed proteins in this process. The major protein component in the seed, the coagulant protein has been implicated to be involved as the key protein responsible for clarifying water.

Although a number of papers are found on the water clarification properties of *Moringa*, only a few deal with heavy metal removal potential of *Moringa*. Minimal studies have been directed towards its sorption behavior of the whole seed powder for the removal of toxic metals from water bodies. Almost no reports are available on the storage proteins which are found in large proportion in these species which may take part in various physiological functions of the plant. There are also only a few reports on the existence of other biologically important proteins from the seeds such as the lectins, glycosidases.

The laboratory where this work has been carried out is working on the purification and characterisation of lectins and glycosidases. Several new lectins with different sugar specificities from plants and animals have been purified employing new affinity methods developed. Furthermore methods have been developed to purify glycosidases from plants and animals and they have been biochemically and immunologically characterized in order to understand their structure-function.

The present study was undertaken to isolate, purify the biologically important proteins from the *M.oleifera* seeds, biochemically characterize them and understand their functions.

Towards achieving these goals, first the coagulant protein was purified to homogeneity. Availability of this protein in large concentrations was a useful tool to characterize this protein biochemically, raise an antibody for this and to develop an immuno-affinity gel for the purification of the coagulant protein. Additionally, its secondary structure properties as well as its thermal stability and metal binding properties were analysed.

Lectins constitute biologically important proteins and have been extensively characterized from the seeds of legumes. Preliminary studies using the crude seed extracts of *M.oleifera* showed hemagglutinating activity with pronase treated rabbit erythrocytes. This provided the lead to first determine the sugar specificity of this protein and to isolate the same employing an affinity matrix developed and to further characterize the lectin in terms of its temperature and pH stability, interaction studies with glycosidases and to determine its fine sugar specificity, and immunological reactivity studies with legume lectin antibodies.

The crude extracts of the seeds also exhibited α-mannosidase and α-galactosidase activities and there is no information on the purification of glycosidases from this seeds. With a long term objective to understand the structure and function of these enzymes, we purified these to homogeneity using conventional chromatographic techniques. Having the enzymes in pure form, provided a useful means to biochemically characterize the same and to understand their properties. Furthermore, their localization in the seeds was also determined.

CHAPTER 2

Further characterization of the coagulant protein from the seeds of *Moringa oleifera*

2.1.0. Introduction

The treatment of water to render it fit for human consumption has become a problem of central importance, both in developing and developed countries. In developing countries, the quality of drinking water is often insufficient and sometimes hazardous to health. Water is particularly vulnerable to contamination from discharge of waste by various industries, among which heavy metals are the most important components. Unlike the organic toxicants, which can be degraded, inorganic metal species are immutable and persist indefinitely in the environment. The non-biodegradable nature and long biological half-life of most of the metals have lead to potential accumulation and exposure to humans via water or food [Sharma *et al.,* 2006]. Water treatment usually comprises water clarification and disinfection.

In developed countries, various synthetic coagulants of aluminum (alum lime, aluminum sulphate, and polyaluminum silico sulphate), ferric salts (iron hydroxide and iron chloride) and soda ash are widely used processes [Boisvert *et al.,* 1997, Nalm *et al.,* 1998; Okuda *et al.,* 2001a] despite the fact that their safety for health during long-term exposure may induce Alzheimer's and other disease [Miller *et al.,* 1984; Martyn *et al.,* 1989]. Recently, there have been many reports of the possible link between high levels of residual aluminum and several medical disorders and this initiated a global interest in the search for a substitute coagulant that will be safer for health. As a result, it is desirable to find sustainable alternatives that are harmless to human health and to the environment.

A number of natural coagulants are known to be effective for water treatment but their application has been limited due to lack of extensive scientific understanding and some draw backs associated with the release of organic and nutrient contents to the treated water. Naturally occurring alternatives to currently used coagulants and disinfectant have been considered, including cultivated plants. Of particular interest are the seeds of a tropical tree, *Moringa oleifera* (MO), as they contain an active coagulating protein traditionally used for the clarification of drinking water in the rural areas of Sudan and Malawi [Okuda *et al.,* 2001 a, b]. It has been reported that MO seed extracts exhibit good coagulation [Olsen, 1987; Jahn, 1988; Muyibi and Evison, 1995].

The turbidity of water often results from the presence of negatively charged particles in a colloidal structure, the clarification of which requires acceleration of the sedimentation rate. For this purpose, positively charged agents are used to form complexes with negative charges of the colloid, a process called coagulation.

Moringa oliefera (MO) coagulant protein can be extracted by water or salt solution (commonly NaCl). The amount and effectiveness of the coagulant protein from salt and water extraction methods vary significantly. In crude form, the salt extract shows a better coagulation performance than the corresponding water extract. This may be explained by the presence of a higher amount of soluble protein due to the salting-in phenomenon [Okuda *et al.*, 1999]. Okuda isolated an active component which is neither a protein nor polysaccharide with a molecular weight about 3000 Da using anion exchange chromatography. This is used for treatment of low turbid waters [Okuda, 2001a].

The seeds from this plant contain active coagulating agents characterized as dimeric cationic proteins having a molecular weight of around 6.5 kDa and with pI greater than 9.6 [Ndabigengesere *et al.,* 1995]. Previous studies have reported that the molecular mass of the protein from MO seed was 6.5-13 kDa and isoelectric point above 10 [Gassenschmidt *et al.,* 1995; Ndabigengesere *et al.,* 1995]. Ghebremichael *et al.,* [2006] employed a single step elution for the MOCP using ion exchange column (IEX). The potential drawback in this procedure is pooling both non-coagulant proteins which elute at low ionic strength together with that of coagulant protein eluted at high ionic strength. Attempts to purify in high yield lead to loss of MOCP.

In a recent study a hemagglutinin with complex sugar specificity was isolated from the seeds of *Moringa oleifera* which was found to have coagulant activity [Uma *et al.,* 2008]. The coagulant protein showed both flocculating and antibacterial effects [Broin *et al.,* 2002; Ghebremichael *et al.,* 2005]. In spite of its nutritional and medicinal importance, the plant still remains underexploited. No attempts were made to look into the biochemical properties of purified MOCP with respect to haemagglutination, antifungal, hemolytic activities.

The present study focused on the isolation and purification of MOCP using cation exchange column together with gel filtration. Purity of the protein was analysed with

electrophoresis. Raising the antibodies and preparing the immunoaffinity matrix for the purification of MOCP in a single step which can be used later used in screening of different biochemical properties of this protein *in vitro*. Further the monospecific antibodies were used for the immunolocalization of this protein in the seeds. Key advantage of the purification is that it reduces the organic load in treatment systems without requiring more complex protein production methods, such as recombinant (heterologous) expression.

2.2.0. Materials and Methods

Seeds of *Moringa oleifera* (Variety: PKM_1) were purchased locally. Sephadex G-75, CM cellulose, FITC conjugated goat anti-rabbit IgG, and the standard proteins used were purchased from Sigma chemical company. All other chemicals and reagents were procured locally from reputed firms and were of high purity.

2.2.1. Coagulation activity test (Qualitative)

Small volume coagulation activity assay on clay suspension was performed according to [Ghebremichael *et al.,* 2005]. First the water sample for the assay was prepared by adding clay (1 g) to 100 mL tap water, stirred for 30 min and allowed to settle for 24 h to allow complete hydration. Desired turbidity was obtained by dilution. Purified coagulant (10 μL; 0.5 mg/mL) was added to 1ml clay suspension and mixed instantly. Absorbance at 500 nm (OD_{500}) was measured after a 1 h settling time. A clay sample without addition of coagulant protein was considered as a blank. A difference in OD_{500} between an alum-treated (5% w/v solution) sample and a blank (clay suspension without coagulant) was defined as 10 coagulation activity units.

2.2.2. Extraction and purification of the *Moringa oliefera* coagulant protein (MOCP)

The seeds obtained were deshelled just before the extraction and the kernel was ground using a kitchen blender. Seed powder was defatted using acetone and the solids were dried at room temperature. The protein from 50 g of seed powder was extracted overnight with 500 mL of 25 mM PBS pH (7.0). The suspension was clarified by centrifugation and filtered through Whatmann paper No. 3. The filtrate is termed the crude extract.

The purification of protein was done using the method of Ghebremichael *et al.*, [2006] with slight modifications described below. The protein was precipitated from

aqueous filtrate by the addition of solid ammonium sulphate ($(NH4)_2SO_4$), to the saturation of 80%. The precipitated protein was separated by centrifugation, re-suspended in 25 mM PBS pH 7.0. The protein solution was dialysed through cellulose dialysis tubing (Himedia) with a molecular weight cut-off of 12-14 kDa. The protein remained inside the dialysis tube and was poured on a carboxymethylcellulose (CM) gel equilibrated with 25 mM PBS pH 7.4 to retain the active protein and elution was carried out with 0.3 M NaCl in 25 mM PBS pH 7.4 followed by 0.7 M NaCl in 25 mM PBS pH 7.4. The 0.7 M NaCl eluted fraction mainly contained the coagulant protein which was concentrated and dialyzed again through the 12-14 kDa cutoff dialysis tubing. Further, to get a homogeneous preparation of the MOCP it is passed through a gel filtration column. Peak fractions containing coagulant protein were pooled and concentrated. The homogeneity of the protein was further confirmed using SDS-PAGE. This purified protein is subjected to lyophilization and a white protein powder was obtained.

2.2.3. Gel filtration and Native Molecular weight determination

The 0.7 M NaCl eluted peak protein fraction after cation ion exchange was further purified using Sephadex G-75 (90 x 1.6 cm) column pre-equilibrated in 25 mM PBS pH 7.0 at 4-8°C. Peak fractions containing coagulant protein were pooled and concentrated. The same column was also used for the determination of native molecular mass of the *Moringa oleifera* coagulant protein (MOCP) after calibrating the column with proteins of known molecular weight, *viz.*, using Bcl –XL from mouse (27.0 kDa) casein (23.0 kDa), myoglobin (16.0 kDa), cytochrome c (12.4 kDa), as reference proteins. 2.0 mL fractions were collected. The protein so obtained was termed as the purified *Moringa oleifera* coagulant protein (MOCP) and used in the subsequent studies. The protein was lyophilized and stored at 4°C until use.

2.2.4. Protein determination

The protein content in the extract was carried out by the method described by Bradford [1976] using bovine serum albumin as standard. During purification process, the protein concentration in the column eluates was routinely checked by measuring the absorbance at 280 nm.

2.2.5. Electrophoresis (Separation of MOCP on Tricine and SDS PAGE)

Tricine-SDS-PAGE was performed according to the method of Schagger *et al.,* [1987] using 18% separating gel. Protein bands were visualized by coomassie brilliant blue R-250 staining. The protein sample was analyzed both in the presence and absence of reducing agents. The standard marker proteins used were molecular weight markers: ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.0 kDa), lysozyme (14.0 kDa) aprotinin (6.5 kDa) and Insulin (3.0 kDa). SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli *et al.* [1970] using 15% or 12.5% separating and 5% stacking gel respectively. The standard marker proteins used were ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.0 kDa), and lysozyme (14.0 kDa). Proteins were detected by staining the gel with coomassie brilliant blue R-250.

2.2.6. Two- dimensional electrophoresis (2DE)

Isolelectric focusing (IEF) was performed with IPG strips pH 3-10 using Ettan IPG Phor-3 Isoelectric Focusing system (GE Healthcare) following the manufacturer's instructions. The sample (100 μg) was mixed with rehydration buffer consists of 0.5% IPG buffer pH 3- 10,8 M Urea, 2% CHAPS and 20 mM DTT (freshly added) and applied on to an IPG strip holder. The IPG strip was gently placed into the strip holder without air bubbles between strip and buffer. After first dimension, the strip was equilibrated in a buffer that consists of 6 M Urea, 75 mM Tris-HCl (pH 8.8), 29.3% (v/v) Glycerol, 2% SDS and freshly added DTT (10 mg/ml), Iodoacetamide (25 mg/ml) separately. The strip was then placed on the surface of the second-dimension gel (12.5% SDS-PAGE gel without sample wells) and sealed with agarose gel. Proteins were stained with Coomassie Brilliant Blue G 250.

2.2.7. Raising antibodies to the purified MOCP

Polyclonal antibodies were raised in a rabbit (weighing 3.0 kg) to the native purified protein. Approximately 500 μg protein was emulsified in complete Freund's adjuvant and administered to a rabbit by subcutaneous injection. Subsequent booster injection was administered with antigen emulsified in incomplete Freund's adjuvant on the 21st day and 34th day. Blood (10-15 mL) was collected from the rabbit by ear vein puncture. After collection, blood was allowed to clot at room temperature. Serum was

separated from the clot and any remaining insoluble materials were removed by centrifugation at 10,000 rpm for 10 min at 4°C. The clear supernatant was stored frozen at –80°C until use.

2.2.8. Preparation of specific IgG against *Moringa oleifera* coagulant protein.

Coupling of the purified MOCP to Affigel-10 was carried out following manufacturer's instructions. Purified MOCP was dialyzed against 0.1 M HEPES buffer pH 7.4. 2.0 mL of Affigel-10 (Bio-rad labs) was thoroughly washed with chilled isoproponal followed by cold-water and 0.1 M HEPES buffer pH 7.4. To this 8 mg/mL MOCP in HEPES buffer, was added and coupling reaction allowed to proceed at 4°C for 24 h by end over end rotation. At the end of this incubation period, the unbound fraction collected, unreacted sites in the gel were blocked with 0.1 M-ethanolamine-HCl pH 8.0 (200 μL/mL Affigel-10) for one hour at 4˚C. The gel was finally washed with PBS and equilibrated with TBS before use. Total antiserum to the purified MOCP (mL) was dialyzed against 10 mM Tris–HCl buffer pH 7.4 containing 150 mM sodium chloride (column buffer) and then applied to the MOCP affigel at 4°C equilibrated with the same buffer. After the gel was extensively washed with column buffer, bound IgG was specifically eluted with six column volumes of 100 mM Glycine-HCl buffer pH 2.6. The eluted protein was immediately neutralized with 2 M Tris, analyzed on a 7.5% SDS-PAGE under non-reducing conditions to identify the IgG band and the protein containing the IgG was stored at 4°C.

2.2.9. Preparation of the immuno-affinity gel (IgG-Affigel)

Affinity purified lectin-specific antibodies obtained above was dialysed against 10 mM HEPES buffer pH 7.4 and was concentrated. Protein (10 mg/mL) was allowed to couple onto 2.0 mL Affigel-10 as described above and the final matrix designated as the immuno-affinity gel.

2.2.10. Immuno-affinity chromatography for purification of seed MOCP

MOCP was extracted as described earlier and the concentrated sample was passed through immuno-affinity gel pre-equilibrated with 25 mM TBS pH 7.4 buffer. After washing the gel with the buffer, bound MOCP was eluted with 0.1 M Glycine HCl buffer pH 2.5 immediately neutralized with 2 M Tris and the absorbance at A_{280} was recorded, the protein containing fractions were pooled, extensively dialyzed against column buffer and analyzed for the presence of MOCP by SDS-PAGE [Nagender and Siva Kumar, 2008].

2.2.11. Western blot analysis

This was carried out according to Towbin *et al*., [1979]. The purified MOCP was separated on a 12.5 % SDS-PAGE and the protein bands transferred to a nitrocellulose membrane. The membrane was blocked with 5% defatted milk powder in TBS buffer pH 7.4 for 1 h. The membrane was incubated with antiserum raised for MOCP as the primary antibody (1:500 dilution) for one hour in TBS buffer pH 7.4 containing 1% defatted milk powder. The membrane was washed with TBS, TBS Tween followed by TBS for 5 minutes each and blocked for 5 min with 5% defatted milk powder in TBS buffer. The membrane was later incubated with secondary antibody (1: 1000 dilution), goat-anti rabbit IgG conjugated with alkaline phosphatase for 1h in TBS buffer containing 1% defatted milk powder. The membrane was washed with TBS, TBS Tween followed by TBS and then with distilled water. The blot was developed with BCIP/NBT (Bangalore Genei, Bangalore) substrate until the bands were visualized and then the blot was washed with water to stop the reaction. The membrane was air dried and photographed.

2.2.12. Immuno-localization studies

The dry seeds were imbibed for 24 h on moistened filter paper. After that the seeds were dehulled and thin transverse paraffin sections of kernel were made using microtone and collected on glass slides as described [Deepak *et al.,* 2003]. The sections were washed with TBS containing 0.2% Tween 20 (TBS-T) for 10 min. The MOCP-specific IgG (1:500 dilutions) obtained for the MOCP was used as a tool to localize the protein in the seeds by confocal laser scanning microscopy. Non-specific sites were blocked with TBS-T containing 3% BSA for 1 h. After washing with TBS-T, tissue sections were treated with MOCP-specific IgG (primary antibody) diluted 500-fold in TBS-T and washed before being treated with secondary antibody (incubation in dark with FITC conjugated goat anti-rabbit IgG using 1000-fold dilution in TBS-T).The slides were washed extensively using TBS-T. All the processing reactions were done at room temperature. For immunofluorescence detection, sections were examined using confocal microscope.

2.2.13. Antifungal assay

The assay for antifungal activity towards species of *Fusarium oxysporum*, *Fusarium solani*, *Botrytis cinerea* and *Aspergillus nidulans* was conducted in 100 mm x 15 mm petri dishes containing 10 mL of potato dextrose agar [Lam *et al.*, 2000]. These fungi have been demonstrated to be sensitive to a variety of antifungal proteins. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the periphery of the mycelial colony. An aliquot (6 µL containing 10 µg) of a coagulant protein was added to a disk. The disks were incubated at 23°C for 72 h until mycelial growth had surrounded disks containing the negative control (*i.e.* buffer only).

2.2.14. Hemagglutination activity

The hemagglutination assay for purified MOCP was performed according to Rameshwaram, N. R. and Nadimpalli, S. K. [2008]. 100 µL of purified MOCP (1 mg/mL) is serially diluted in 100 µL of phosphate buffer saline pH (7.4) in an agglutination plate. About 100 µL of 4% suspension of rabbit red blood cells (trypsin treated and pronase treated) were added in agglutination plate, incubated at 37^{o}C for 1 h and the hemagglutination was observed. PBS and blood was used as a negative control.

2.2.15. Trypsin inhibitory activity

Trypsin inhibitory activity was measured using α-*N*-benzoyl-l-phenylalanine-l-valine dl-arginine-*p*-nitroanilide (Bz-Phe-Val-Arg-NA; Sigma) as a substrate [Tunlid *et al.*, 1994]. Purified MOCP was pre-incubated with trypsin (Sigma) at room temperature for 1 h. Then the substrate (Bz-Phe-Val-Arg-NA) was added into the mixture and the residual activity was measured by the method previously described [Yang *et al.*, 2001].

2.2.16. Hemolytic activity

It was performed by using rabbit and human erythrocytes [Park *et al.*, 2007]. 50 µL MOCP of different concentration was incubated with 50 µL of PBS (25 mM) to this 4% rabbit erythrocyte suspension was added, and incubated for 30 min at room temperature. After centrifugation at 1,500 x *g* for 5 min, the absorbance of the supernatant solution was measured at 540 nm. RBCs in PBS (A_{blank}) and in 0.1%

Triton X-100 (A_{triton}) were used as the negative and positive controls, respectively. The percent hemolysis was calculated according to the equation:

% Hemolysis= [(A_{sample}-A_{blank})/ (A_{triton}-A_{blank})] x 100.

2.2.17. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan, website: http://www. jascoint.co.jp) equipped with a Peltier thermostat supplied by the manufacturer. Samples were placed in a 2-mm pathlength rectangular quartz cell. MOCP concentration was 0.1 mg/mL for measurements in the far UV region (250-190 nm). In order to study thermal unfolding of the protein, CD spectra were recorded in the far UV region at different temperatures. In addition, temperature scans were performed between 30 and 100 °C at a scan rate of 1° per minute, by monitoring spectral changes at (ellipticity (Θ)) 222 nm, for far UV regions, which is a determinant for the secondary structure. Influence of the pH on the structural changes was also studied by incubating the MOCP at different pH range.

2.3.0. Results

2.3.1. Extraction and purification of MOCP

MOCP is purified to homogeneity using both cation ion exchange and gel permeation chromatography. The first step was to purify the protein owing to its cationic nature, the choice of purification was by cation-exchange chromatography, where the MOCP bound to the resin, and was eluted using 300 mM NaCl in 25 mM PBS pH 7.4 followed by 700 mM NaCl in 25 mM PBS pH 7.4. Two peaks were observed peak 'a' and peak 'b' [Figure: 2.1-A]. MOCP was mainly observed in the 700 mM NaCl eluted fraction, as evidenced by the assay of coagulation activity. Peak 'a' did not show any coagulation activity while peak b showed this activity [Figure: 2.1-B]. Several protein bands were observed in peak 'a' whereas, distinct protein band was observed in peak 'b' eluted at 700 mM elution when analysed with 12.5 % SDS-PAGE [Figure: 2.2]. The peak 'b' fraction which contains coagulant protein was pooled and concentrated. This is when subjected to gel filtration (G-75) for removing minor contaminants gave a single predominant peak corresponding to 13 kDa [Figure: 2.3-A]. The same column is used for determining the precise molecular mass of the protein after calibrating with standard proteins [Figure: 2.3-B].

2.3.2. Molecular characterization of MOCP

From the total protein (50g extract, 8506 mg protein) by CM cellulose chromatography, 430 mg protein was obtained. In total by gel filtration, 350 mg protein was obtained. The purified protein was subjected to Tricine-PAGE under reducing and non-reducing conditions, as described under methods. Under reducing conditions the protein dissociated into monomeric subunits having same molecular mass and appear as single band of molecular mass of 6.5 kDa (lane 1) [Figure: 2.4]. Whereas, under non-reducing conditions, the protein migrates into single band corresponding to a molecular mass of 13 kDa (lane 2) [Figure: 2.4]. pI value as determined by IEF was >10.

Polyclonal antibodies were raised in a rabbit to the native purified protein. To obtain the specific IgG from whole serum, the antiserum was passed through the MOCP-affigel as described under methods. The specific IgG was eluted from the immuno-affinity gel [Figure: 2.5-A]. Specific IgG was confirmed by SDS-PAGE (7.5%) [Figure: 2.5-B]. The specificity of the antiserum was confirmed in a western blot

experiment [Figure: 2.5-C]. The specific IgG was coupled to affigel-10 and was used as an immuno-affinity gel for the purification of the seed MOCP as described under methods. Typically from 10g of the seed extract about 90 mg of the protein could be obtained by this method. High purity MOCP was eluted using the immuno affinity gel [Figure: 2.6-A]. Figure: 2.6-B is the 15 % SDS-PAGE of the immuno affinity eluted protein.

2.3.3. Immunolocalization of MOCP in the seed kernel

In order to study the role of the MOCP in plant we used immunolocalization studies to trace this protein, which is present in large quantities in dried seeds. By incubating the thin sections of the *Moringa oleifera* seed kernel with the specific IgG, and using FITC conjugated secondary antibody, it was shown that the protein was localized to the vacuoles [Figure: 2.7].

In order to investigate whether the purified MOCP exhibits any other biochemical properties such as the trypsin inhibitory activity, hemagglutination activity, and hemolytic activity, these were assayed and the results suggests that this protein does not have any of these above said properties.

2.3.4. CD spectroscopy and secondary structure

Circular dichroism spectra of native MOCP in the far UV region is shown in Figure: 2.8. The far UV CD spectrum of native MOCP showed two negative peaks at 222 nm and 208 nm. In order to estimate the content of different types of secondary structures in MOCP, the far UV CD spectrum of the native protein in the wavelength range 250–190 nm has been analyzed by the CDSSTR program using the routines available at DICHROWEB (www.cryst.bbk.ac.uk/cdweb/html) [Compton *et al.*, 1986; Lobley *et al.*, 2002]. The values obtained for the different types of secondary structures are: α-helix (59%), β-sheet (13%), and unordered structures (28%). Initial attempts to crystallize this protein in order to get information on its 3-dimensional structure was not successful due to interference of salt crystals with that of protein crystals.

2.3.5. Thermal stability and unfolding of MOCP

To investigate the thermal unfolding of MOCP, Far UV CD spectra of the MOCP were recorded at different temperatures. Spectra recorded at 30, 40, 50, 60, 70, 80, 90 and 100 °C are shown in Figure: 2.9-A. While only moderate changes in the spectral features are seen in the spectra recorded at 30 and 90°C, including small decrease in

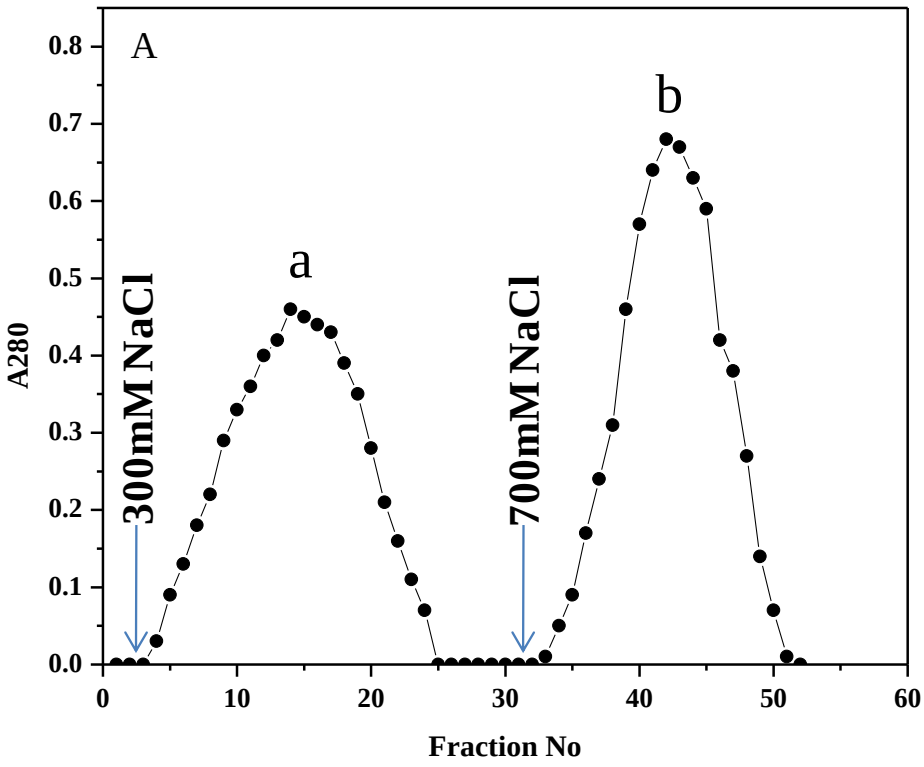
the spectral intensity at 222 nm [Figure: 2.9-B]. The unfolding temperature of ~100 °C indicates that MOCP is a rather stable protein.

2.3.6. pH stability of MOCP

To check the effect of pH on the spectral changes in the far UV region of MOCP, it is incubated with different pH in the range of 2-12 pH. No change was observed in the pH range from 2-10 and changes were noticed only beyond pH 10 [Figure: 2.10 A & B].

FIGURES

Figure: 2.1 A & B


A
a
b
300mM NaCl
700mM NaCl
A280
Fraction No
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
10
20
30
40
50
60

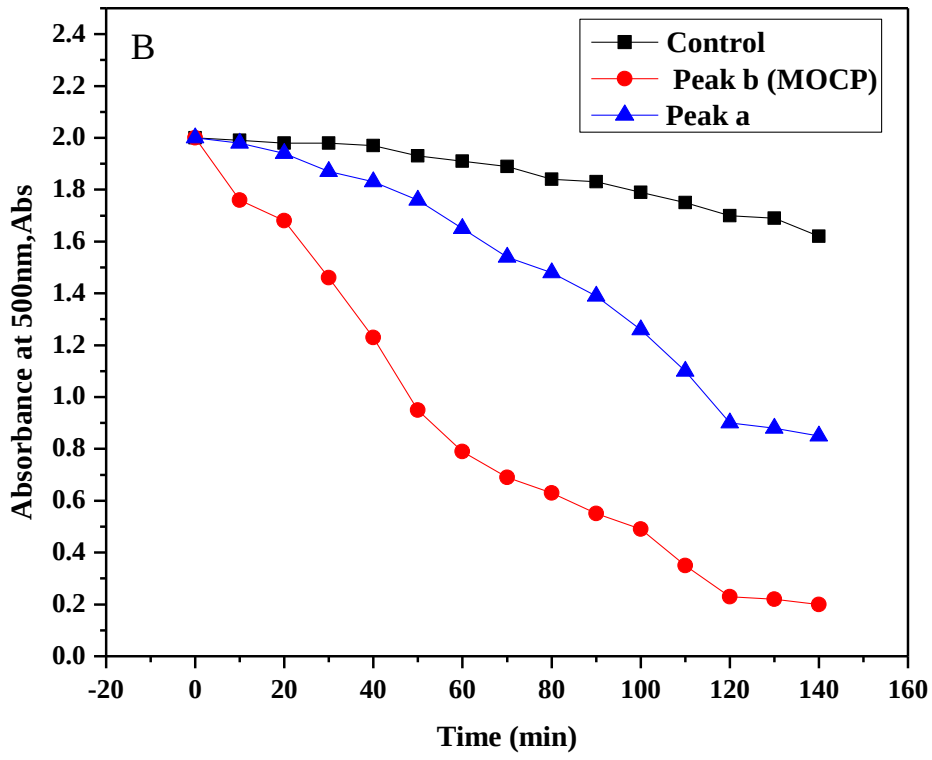

B
Control
Peak b (MOCP)
Peak a
Absorbance at 500nm,Abs
Time (min)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
-20
0
20
40
60
80
100
120
140
160

Figure: 2.1 A & B.

(A) CM- cellulose profile of coagulant protein. The ammonium sulphate fraction (0-100%) obtained from the crude extract was passed through CM-cellulose (15 cm x 2 cm) pre-equilibrated with 25 mM phosphate buffer pH 7.4 (column buffer). The bound protein was step wise eluted with 300 mM NaCl and 700 mM NaCl in the phosphate buffer pH 7.4. Arrows indicates point of application of NaCl.

(B) Coagulation Activity test. The peak eluted protein from the CM-Cellulose column was tested for coagulation activity as described in methods (2.2.1) during the course of 2 h. and measured the absorbance at 500 nm.

Figure: 2.2

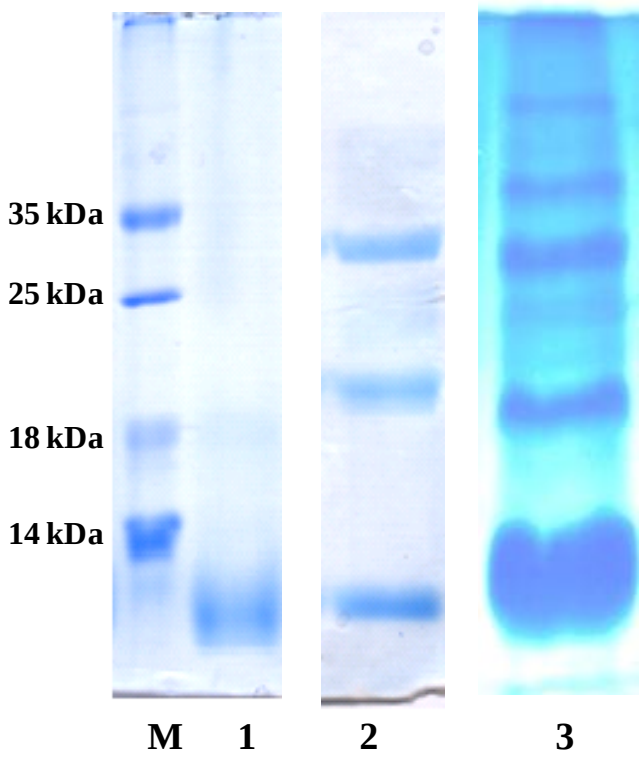
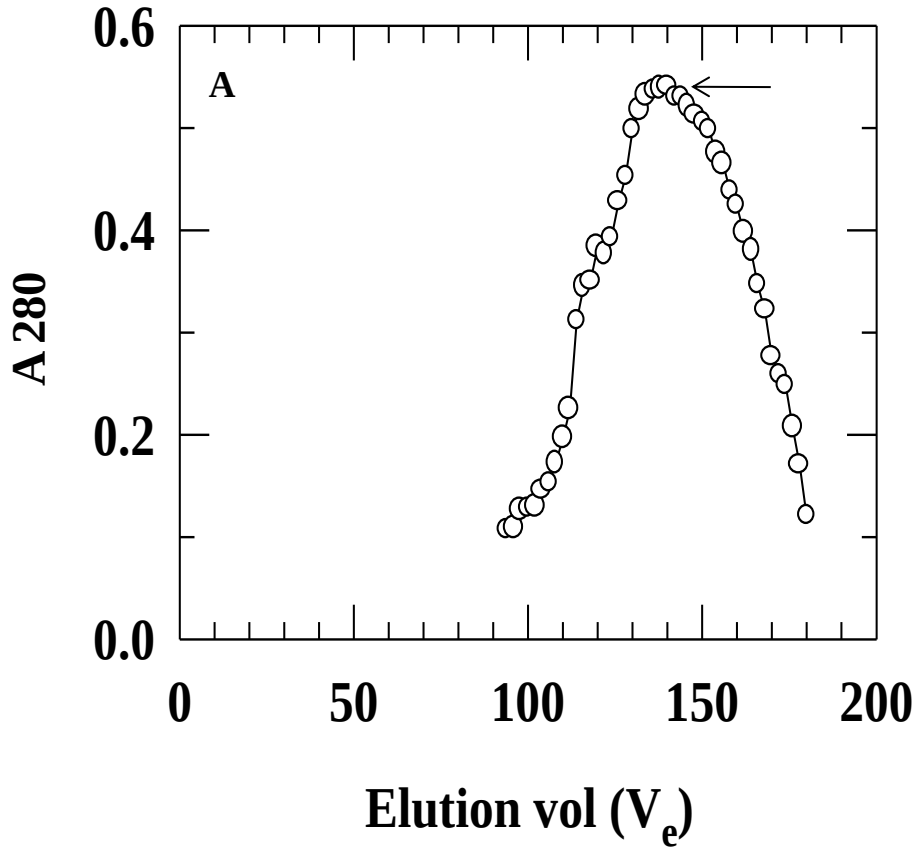


Figure: 2.2. 12.5% SDS-PAGE analysis of the CM eluates: Lane M. Standard marker, Lane 1. Sample from peak ‘b’ fraction (MOCP), Lane 2. Protein from peak ‘a’ fraction, Lane 3. Crude protein.

Figure: 2.3 A & B


A
A 280
0.6
0.4
0.2
0.0
0
50
100
150
200
Elution vol (V_e)

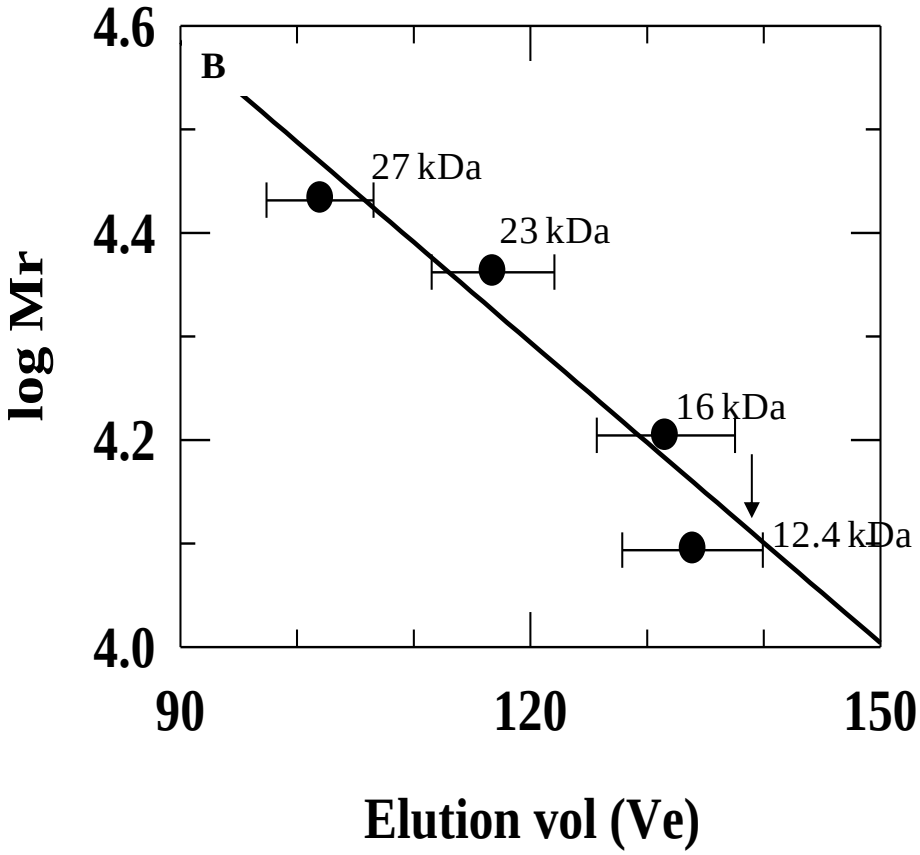

B
log Mr
4.6
4.4
4.2
4.0
27 kDa
23 kDa
16 kDa
12.4 kDa
90
120
150
Elution vol (Ve)

Figure: 2.3 A & B:

(A) Elution profile of the purified MOCP on Sephadex G-75 gel. Aliquot of the MOCP obtained in peak 'b' after CM cellulose chromatography was passed on a Sephadex G-75 (90 cm x 1.6 cm) column pre-equilibrated in 25 mM phosphate, 0.9% NaCl pH 7. 2mL fractions were collected and monitored at A_{280}.

(**B) Calibration of the gel filtration column.** The following standard proteins were used: Bcl –XL from mouse (M_r 27 kDa) casein (M_r 23 kDa), myoglobin (M_r 16 kDa), cytochrome c (M_r 12.4 kDa), as reference proteins. Arrow indicates position of the MOCP eluted.

Figure:
2.4

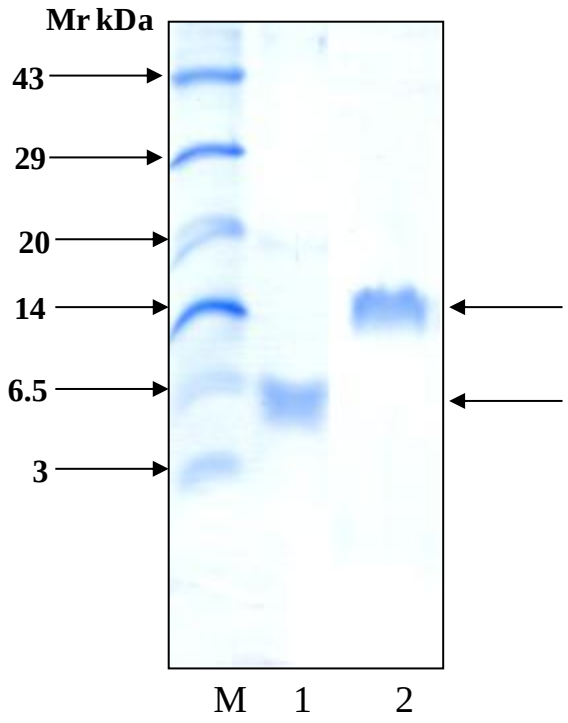
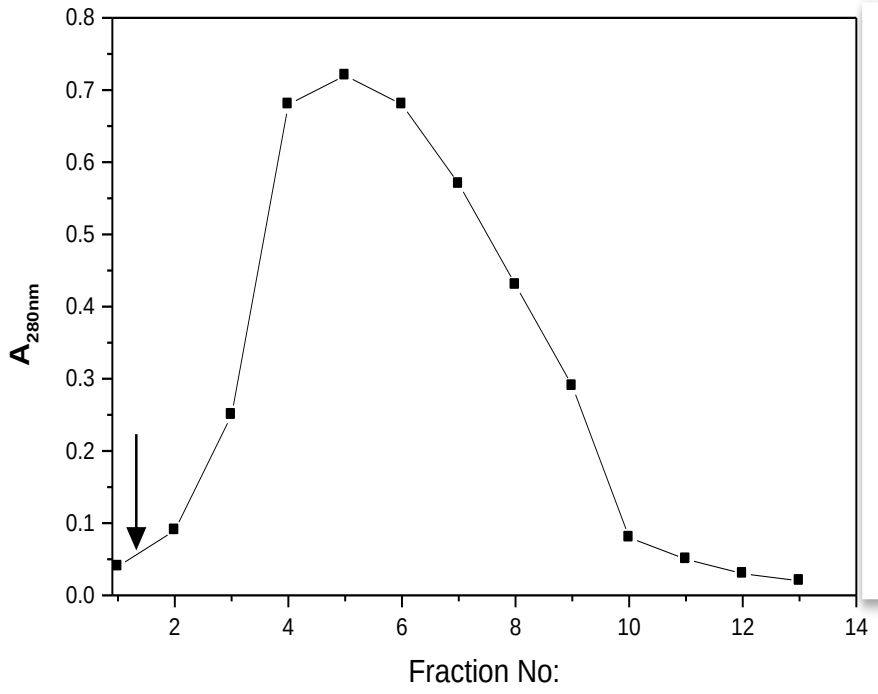
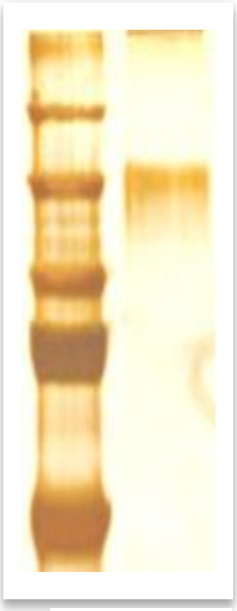


Figure: 2.4. Tricine-PAGE (18%) analysis of the MOCP. Lane M: Molecular weight markers: ovalbumin (M_r 43 kDa), carbonic anhydrase (M_r 29 kDa), soyabean trypsin inhibitor (M_r20 kDa), lysozyme (M_r 14kDa) aprotinin (M_r 6.5 kDa) and Insulin (M_r 3 kDa); Lane 1: Sephadex G- 75 eluate of MOCP under reduced conditions; Lane 2: Sephadex G-75 eluate of MOCP under non-reducing conditions. The proteins were detected by staining the gel with coomassie brilliant blue R-250. Arrow indicates the position of the MOCP under reducing and non-reducing conditions showing the molecular mass of 6.5 kDa and 13 kDa respectively.

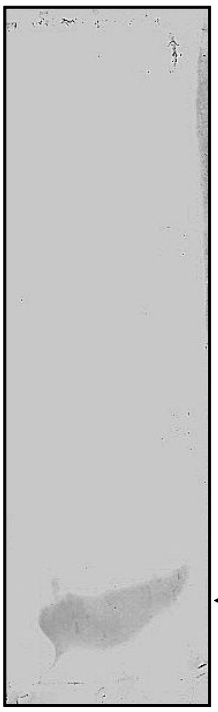
Figure: 2.5 A, B & C



A



B



C

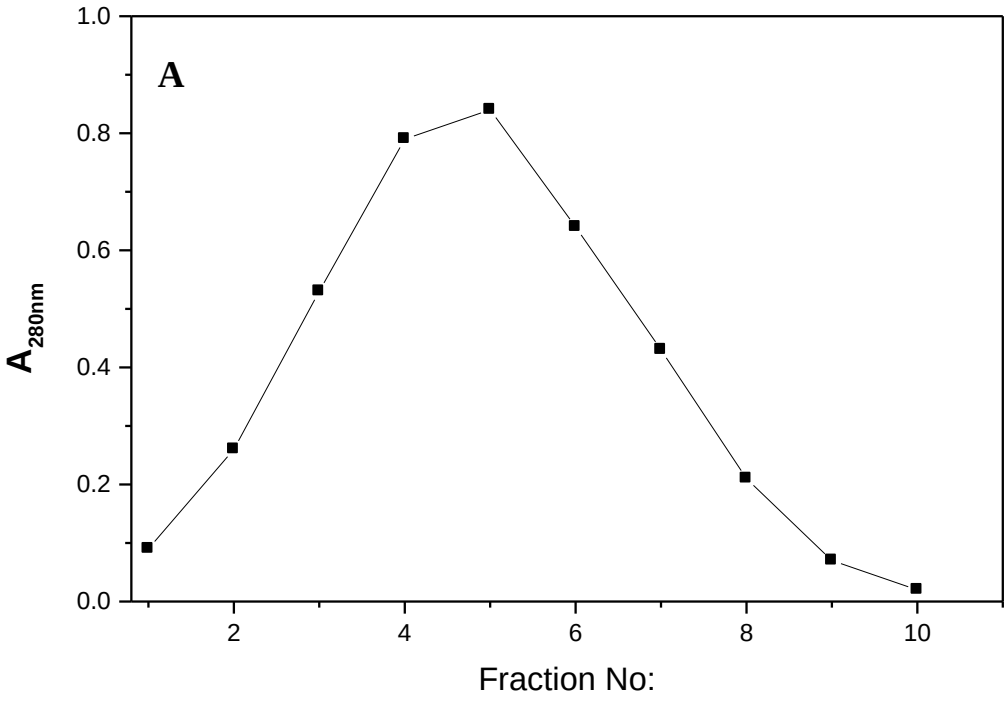
Figure: 2.5A, B & C.

(A) Elution profile of MOCP-specific IgG from MOCP- affigel. Anti-MOCP antibodies from the antiserum to MOCP were purified on MOCP-affigel equilibrated with 10 mM Tris–HCl buffer pH 7.4 containing 150 mM sodium chloride (column buffer). After the gel was extensively washed with column buffer, bound IgG was specifically eluted with 0.1 M Glycine-HCl buffer pH 2.65. The eluted protein was immediately neutralized with 2M Tris. Arrow indicates point of application of the elution buffer.

(B) 7.5% SDS-PAGE analysis of IgG. Lane 1. Protein marker, Lane 2. MOCP specific IgG indicated by arrow.

(C) Western blot analysis of MOCP. Purified MOCP was separated on 12% SDS-PAGE, transferred to NC membrane, incubated with specific IgG, followed by incubation with goat anti-rabbit IgG ALP conjugate (1:1000). Blot was developed using BCIP/NBT reagent.

Figure: 2.6 A & B


A
1.0
0.8
0.6
0.4
0.2
0.0
A_{280nm}
2
4
6
8
10
Fraction No:

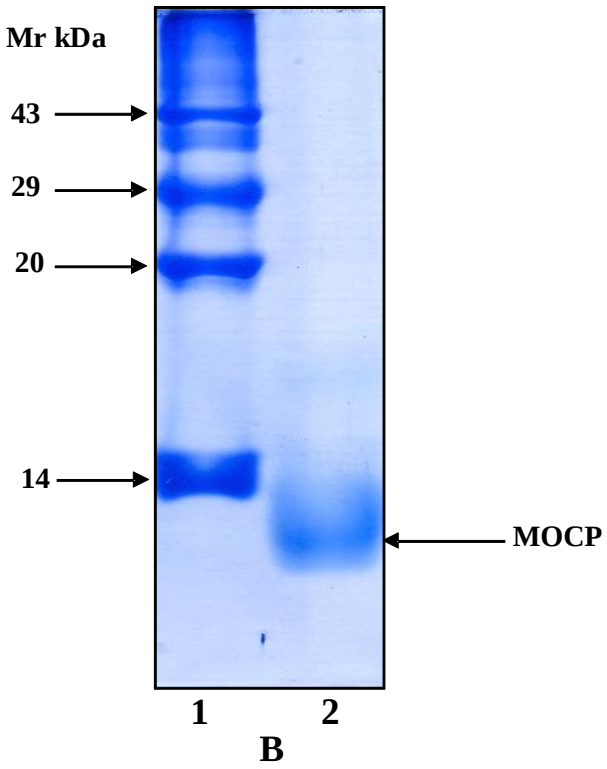

Mr kDa
43
29
20
14
MOCP
1
2
B

Figure: 2.6A & B: (A) Immuno-affinity purification profile of MOCP. **(A)** MOCP specific antibodies were coupled to Affigel-10 in presence of 0.1 M HEPES buffer. MOCP was extracted as described earlier and the concentrated sample was passed through immuno-affinity gel pre-equilibrated with 25mM TBS pH 7.4 buffer. After washing the gel with the buffer, bound protein was eluted with 0.1 M Glycine-HCl buffer pH 2.65 and absorbance at A_{280} was recorded. **(B) 15% SDS-PAGE of the eluted MOCP:** Lane 1. Standard protein marker and Lane 2. Immuno-affinity purified MOCP under reducing conditions.

Figure: 2.7.

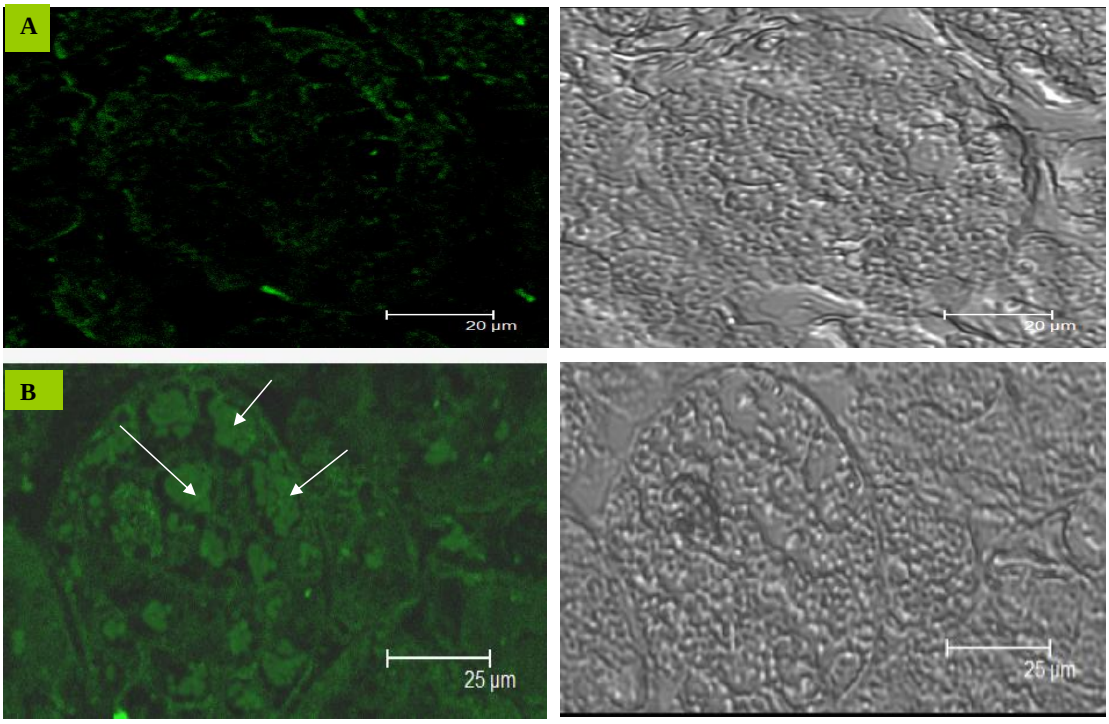
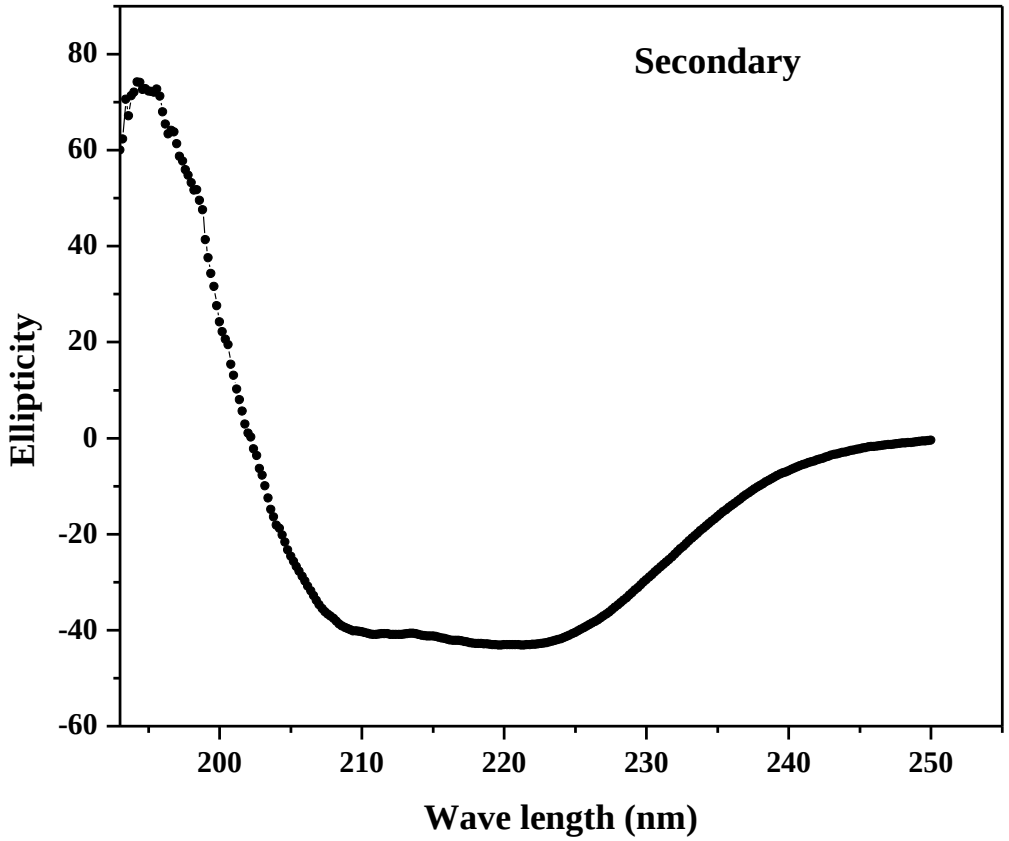


Figure: 2.7. Immunolocalization studies. MOCP kernel transverse sections, **(A)** probed with pre-immune serum and **(B)** probed with MOCP antiserum (1:500 dilutions) followed by incubation with FITC conjugated secondary antibody (green)(1:1000 dilutions). Arrows indicate the abundant MOCP identified. The images against A and B are the corresponding transmission images. Bar in the top panel indicates 20 µm, bottom panel 25 µm. Arrows indicate MOCP localized in vacuoles.

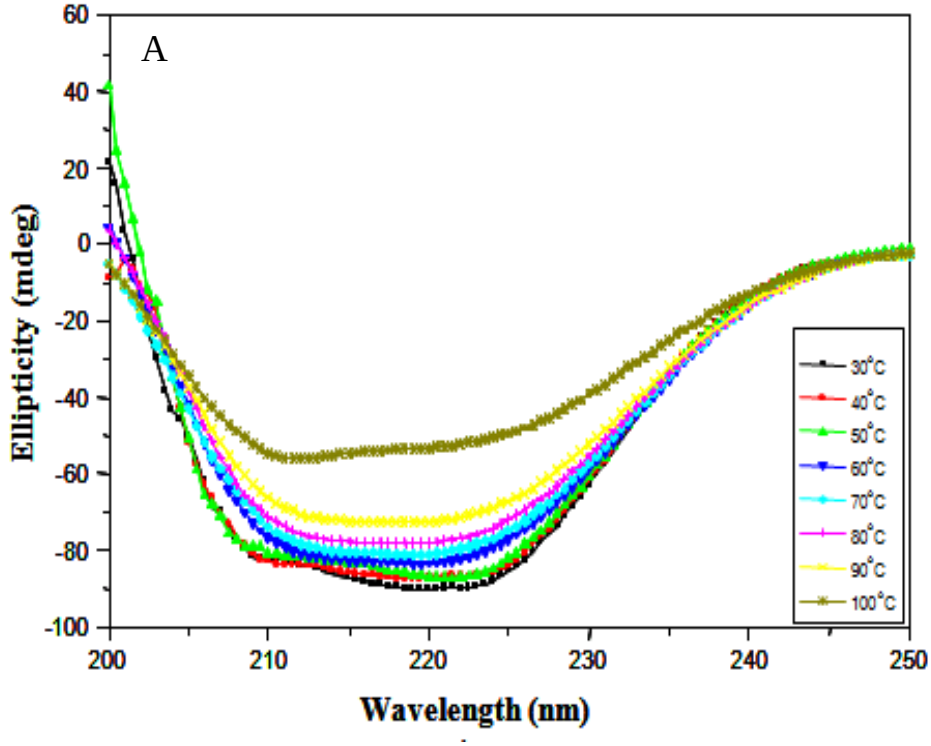
Figure: 2.8.



Secondary structure	α-helix	β-sheets	Unordered structures
MOCP	59%	13%	28%

Figure: 2.8. Secondary structure of MOCP. Far UV-CD spectrum (250-195 nm) of the MOCP (0.1 mg/mL) in distilled water at 30°C. The structure parameters were analyzed by CDSSTR program (DICHROWEB).

Figure: 2.9 A & B


A
Ellipticity (mdeg)
Wavelength (nm)
30°C
40°C
50°C
60°C
70°C
80°C
90°C
100°C

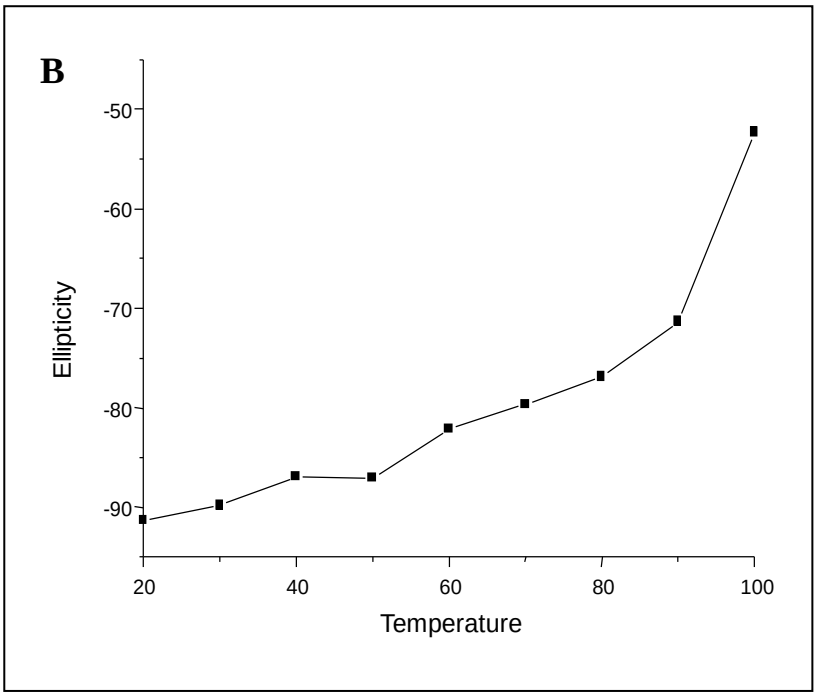

B
Ellipticity
Temperature

Figure: 2.9 A & B. Effect of temperature on the CD spectrum of MOCP.

(A) Far UV CD spectra (200-250 nm) of MOCP at different temperatures. Spectra were recorded at 30,40,50,60,70,80,90 and 100°C.

(B) Ellipticity (Θ) at 222 nm.

Figure: 2.10 A & B

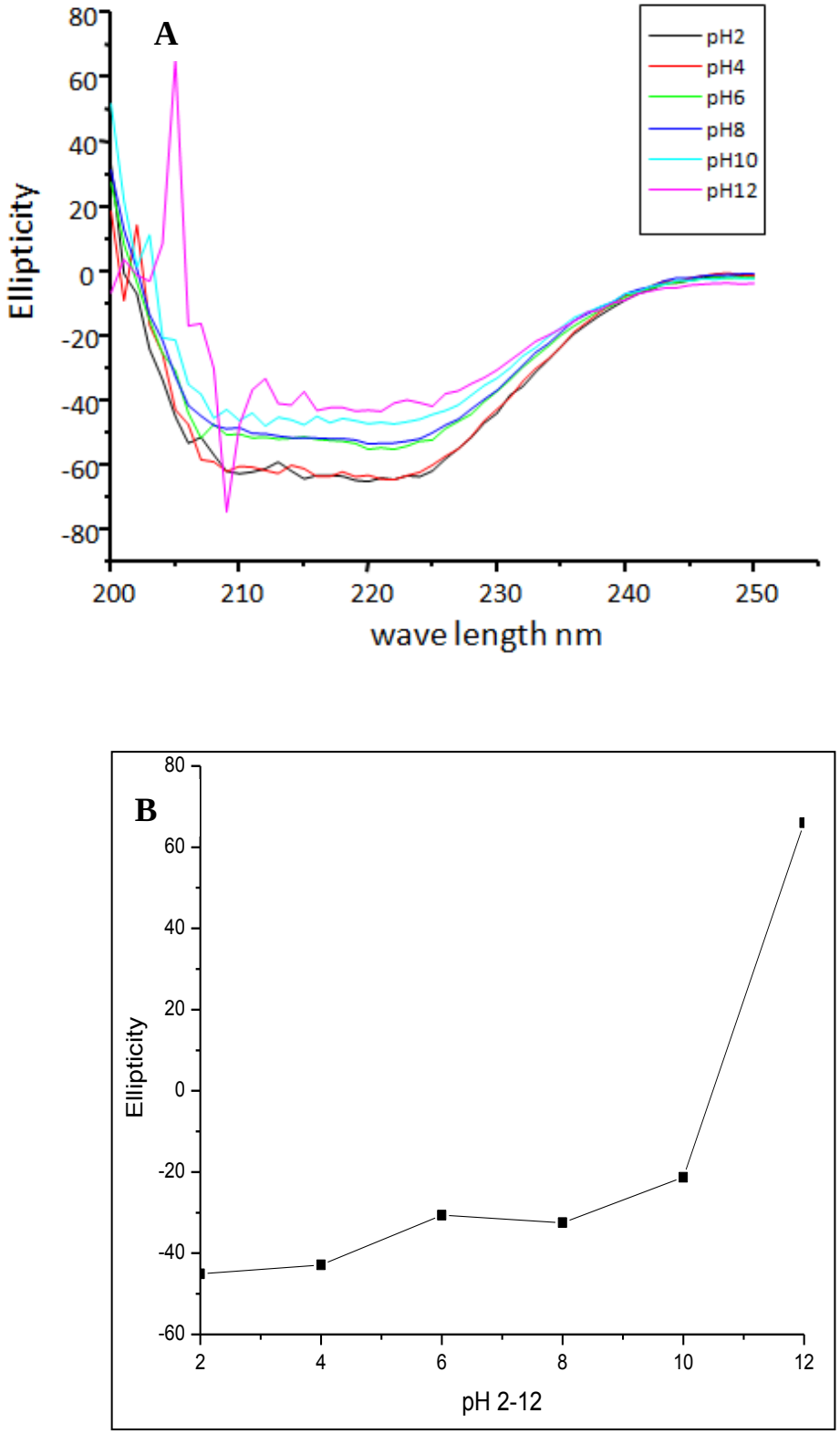


Figure: 2. 10 A & B. Effect of pH on the secondary structure of MOCP:

(A) Far UV CD spectra (200-250 nm) of MOCP at different pH. Spectra are recorded at 2, 4, 6, 8, 10 and 12 pH.

(B) Ellipticity (Θ) at 222 nm.

2.4.0. Discussion

The present study was undertaken to further characterize the MOCP in order to get new information on its molecular properties, immunological properties and structural properties. Owing to the cationic nature of the MOCP CM-cellulose cation exchange chromatography was employed to purify this protein. The protein was completely adsorbed on the column with 25 mM PBS pH 7.4. The bound proteins were eluted in two steps. In the 700 mM elute coagulant protein was observed. Single ion exchange chromatographic step itself is enough for obtaining maximum MOCP. However, minor high molecular mass proteins are present which hinder in the high purity of the sample. Earlier reports on purification of this coagulant protein ignored this condition and single step elution fraction was used for studying the coagulation properties [Ghebremichael *et al.,* 2006]. From figure: 2.3-A it is evident that the protein is homogeneous, and the native molecular mass was found to be 13 kDa [Figure: 2.3-B]. The protein under non-reducing conditions, migrated as a single protein band corresponding to a molecular mass of 13 kDa [Figure: 2.4], and in Tricine-PAGE, under reducing conditions, the protein dissociated to give a single band corresponding to a molecular mass of 6.5 kDa and under non-reducing conditions the protein migrated as a single band corresponding to a molecular mass of 13 kDa. These results suggest that the native protein is a homodimer and is linked by disulfide bridges, consistent with the earlier findings [Ghebremichael *et al.,* 2005].

Though some other properties of the MOCP have been investigated by other researchers, to date there has been no study on raising an antiserum to the MOCP which can be used to obtain high purity protein in a single step from the crude extracts. Therefore we obtained a polyclonal antiserum for MOCP. The main focus of this is four fold. First, the specific IgG that can be obtained from the antiserum raised for the purified MOCP. Second it would be a useful tool to study the localization of the MOCP in the seed kernel, third the antiserum can be used as an efficient tool to develop ELISA method to quantify the proteins in other parts of the plant and to further identify the precise localization of the MOCP in the seeds and fourth, it can also be used to identify any protein-protein interactions between MOCP and other proteins in the plant. The specificity of the antiserum was confirmed in a western blot experiment.

It is noteworthy that studies on the subcellular localization of MOCP revealed that the coagulant protein accumulated in storage vacuoles present in abundance in dry seeds. This signifies that coagulant protein is a storage protein. By incubating the thin sections of the *Moringa oliefera* seed kernel with the specific IgG, and using FITC conjugated secondary antibody, it was shown that the protein was localized to the vacuoles. In literature, the localization of several plant proteins was done using antibodies to the proteins.

Proteins isolated from some medicinal plants exhibit haemolytic activity along with the antibacterial and antifungal activities [Thirunavukkarasu *et al.,* 2011]. Saline extract of the *Enterolobium contortisiliquum* seeds contain a protein called Enterolobin which exhibit the hemolytic activity [De Sousa and Morhy, 1989]. *Moringa oleifera* kernel and extracted-kernel samples showed hemolytic activity [Makkar and Becker, 1997]. Since the MOCP was already found to have antimicrobial activity, we tested if it also has hemolytic activity, but we did not detect any hemolytic activity.

In a separate study a large molecular mass protein of approximately 66 kDa with coagulation activity on soil suspension has been purified using cation and anion exchange chromatography methods. The coagulation activity of the protein is comparable to synthetic coagulant alum and natural cationic peptides from the seeds of *Moringa oleifera* as tested on soil suspension, a natural turbid water. The purified protein does not have antimicrobial activity unlike cationic peptides isolated from same source which have both coagulation and antimicrobial activity [Agrawal *et al.,* 2007].

In a recent study a hemagglutinin with complex sugar specificity was isolated from the seeds of *Moringa oleifera* and was found to possess coagulation activity [Uma *et al.,* 2008]. It shares many similarities with this coagulant protein. It is well documented that several seed lectins when orally fed to rats suggest that it is detrimental to the health [Oliveriva *et al.,* 1994; Pusztai *et al.,* 1988] leading to impaired growth and alterations of key organs, particularly the small intestine. Presently, however, it is confirmed that the coagulation activity detected with lectin is different for the coagulant protein.

Biophysical characterization of a purified protein gives valuable information with respect to the stability of the protein and its secondary structure. To understand some of these properties, we carried out specific studies on the purified MOCP for which no earlier information is known. Figure: 2.8 of far UV CD spectrum showed two negative peaks at 222 nm and 208 nm, suggesting the predominance of helical structure along with other secondary structural elements which is the characteristic feature of helical structure proteins.

No information related to the structural thermostability and pH stability of MOCP was reported. MOCP exhibits high thermal stability, changes in the CD spectrum is only seen at high temperatures. Likewise, the secondary structure is also stable under the influence of pH. This information on the MOCP has now provided the basis to study the stability of the MOCP in greater detail and also understand the role of the disulphide bridge in maintaining the ellipticity in the unfolded state. Experimentation using Differential scanning calorimetry would give more information about the transition state as well as the percentage of the reversibility.

In conclusion, the present study provided a highly purified MOCP. It has also been possible to develop a simple immunoaffinity purification method for the coagulant protein from the *Moringa oleifera* (MO) seed in order to overcome the drawbacks of organic and nutrient release. Furthermore, this method directly yields the coagulant protein without having to separate the coagulant protein first by ion exchange chromatography followed by gel permeation chromatography.

CHAPTER 3

Isolation of a galactose specific lectin from *Moringa oleifera* seeds (MoSL): Affinity purification and biochemical characterization

3.1.0. Introduction

Lectins constitute a class of proteins which possess at least one non catalytic domain capable of specific recognition and reversible binding to carbohydrate [Peumans and Van Damme, 1995]. They are ubiquitously distributed in nature and most abundant in the Plant kingdom, where they are found predominantly in the seeds, leaves, barks, bulbs, rhizomes, roots and tubers depending on the plant species [Hankins *et al.*, 1988; Diaz *et al.*, 1990; Zhu *et al.*, 1996; Wright *et al.*, 1999; Van Damme *et al.*, 2000]. Lectins, by virtue of their exquisite sugar specificities are useful tools in widespread applications for monitoring the expression of cell-surface carbohydrates as well as for the purification and characterization of glycoconjugates [Goldstein and Poretz, 1986; Sharon and Lis, 1989; Cummings, 1997]. As a result of these studies, many plant lectins have become a very popular class of proteins because of their obvious potential in aiding researchers in various areas of biological sciences. Regarding their endogenous cellular functions, plant lectins appear to exert multiple physiological roles (described in Chapter 1). However, the majority of the studies on lectins have been carried out on legume species [Kocourek, 1986; Lakhtin, 1994] particularly from their seeds where they comprise up to 15% of the total protein. Extensive study of sequence homology and 3-D structure of various plant lectins suggests that they are conserved throughout evolution and thus may play, yet unknown, important physiological roles [Barondes, 1981; Etzler, 1992].

Research in the laboratory where this work was done is focused on developing new affinity methods for the large scale purification and characterization of biologically important proteins such as the lectins, glycosidases, receptors, with a long term objective to understand their structure and function. In view of their high potential as tools in biochemical and biomedical research, both for preparative and for analytical purposes, we are looking for new lectins in the tropical and subtropical flora that might have special properties in terms of sugar-binding and specificity and /or stability. *Moringa oleifera* (MO) (family: *Moringaceae*) is a tropical plant having many medicinal values which is largely grown in India [Fuglie, 1999]. The seeds contain high amounts of reserve storage proteins (MOCP), which has been used to purify water and has also been characterized in our laboratory [Tejavath and Nadimpalli, 2010]. Previously, there are reports on the identification and isolation of water soluble *M.oleifera* Lectin (WSMoL) acidic protein which is mainly active at pH

(4.5) [Santos *et al.,* 2005]. Moreover, other hemagglutinin proteins; coagulant MoL (cMoL) and MoL were reported from the same plant, which have distinct sugar specificity and molecular properties [Katre *et al.,* 2008; Santos *et al.,* 2009]. A recent study provided evidence that lectins from *Moringa oleifera* were associated with the insecticidal activity [Coelho *et al.,* 2009; Oliveira *et al.,* 2010]. In view of the diversified results on the lectins in literature [Ahuja *et al.,* 2007], it would be also interesting to study the fine sugar specificity of this lectin and naturally occurring and synthetic sugars have been used by us for this study.

The present investigation has been devoted to purify and characterize a D-galactose-binding ***M****oringa* ***o****leifera* **S**eed **L**ectin (MoSL) from seed kernels using galactose affinity gel. The strategy adopted was to prepare an affinity matrix that contained covalently linked galactose (Serlaose-divinyl sulfone-galactose) and use this for the purification of the *Moringa oleifera* seed lectin. This matrix was earlier successfully used by us for the purification of galactose-specific lectins from *Dolichos lablab* seeds, as well as from leaves and roots of the plant [Latha *et al.,* 2006; Rao and Nadimpalli, 2007]. The purified *Moringa oleifera* lectin was further characterized with respect to its glycoprotein nature, biological activity, and sugar specificity. These properties were compared to other known lectins in literature. Furthermore, a lectin-affigel was prepared to study the interaction of the glycosidases from the same plant with the lectin.

So, the present report also deals with the studies of interaction of synthetic glycoconjugates possessing hydrophobic moieties. The studies include the agglutination properties of MoSL by a variety of synthetic glycoconjugates modified with aromatic moiety as described by Kumar *et al.,* [2010].

3.2.0. Materials and methods

3.2.1. Materials

Dry mature seeds of *Moringa oleifera* (MO) PKM $_1$ variety were purchased locally. Synthetic sugars were kindly provided by Prof. C. P. Rao, IIT Mumbai. Seralose 6B, Sephadex G-150, divinyl sulfone (DVS), other chemical modification reagents used in the present study were of high purity and obtained from reputed firms.

3.2.2. Erythrocyte preparation

This was carried out according to Kumar and Rao [1986]. By ear vein puncture, rabbit blood was collected into Alsevier's solution. It was centrifuged at 3000 rpm for 10 min at 4°C. The sedimented erythrocyte pellet was washed thrice with 0.9% saline and the pellet was made to 4% suspension with saline. Processed erythrocytes were treated with 0.05 % (w/v) pronase and incubated at 37°C for one hour. After pronase treatment the erythrocytes are washed to remove the pronase and the erythrocytes were made to 4% using saline.

3.2.3. Hemagglutinating Activity

This was performed in a plexiplate. Lectin samples (100 µL) were serially diluted in 100 µL of physiological saline (0.15 M NaCl). To each well 100 µL of 4% pronase treated rabbit erythrocytes. This reaction was incubated at 37°C for one hour and the hemagglutination was visually observed. Hemagglutination unit (HU) was expressed as the reciprocal of the highest dilution showing detectable agglutination of erythrocytes. The specific activity was calculated as the HU per mg protein (HU/mg). This pronase treated rabbit erythrocytes were used for the hemagglutination tests throughout this work unless otherwise stated.

3.2.4. Preparation of the chromatographic matrix (Seralose-DVS-Galactose-affinity gel)

Seralose-Galactose affinity column was prepared by activating Seralose-6B with divinyl sulfone as described by Latha *et al.*, [2006]. 25 mL of Seralose 6B was washed thoroughly with double distilled water on a sintered glass funnel and the wet cake was suspended in 0.5 M Na_2CO_3 buffer pH 11.0 in a falcon tube. 2.5 mL of DVS was added and the gel suspension rotated for 70 min at room temperature followed by washing with double distilled water. It was later washed with 0.5 M Na_2CO_3 buffer pH 10. Then, 5 g (20% w/v) of galactose in 20 mL of pH 10.0 buffer was added and rotated for 72 h at 4°C. The gel was washed with water and suspended in 0.5 M $NaHCO_3$ pH 8.5. To this, 0.5 mL of β-mercaptoethanol was added to block the unreactive sites and incubated for three hours at room temperature. Finally, the gel was washed with double distilled water and suspended in TBS until further use.

3.2.5. Extraction and purification of the lectin

All operations were done at 4°C unless otherwise mentioned. Freshly dehulled *Moringa oleifera* seeds were ground into a fine powder. The flour was defatted using acetone and air dried at room temperature. The powder obtained was extracted with 0.15 M NaCl overnight. The crude slurry was centrifuged at 12000 rpm for 25 min. Solid $(NH4)_2SO_4$ was added to the supernatant to attain 75% saturation, stirred for 2 h. The precipitate was collected by centrifugation (12000 rpm, 25 min), dissolved in a small amount of saline, and dialyzed extensively against saline. The dialyzed sample was centrifuged as above and the clear supernatant was subjected to affinity chromatography.

3.2.6. Affinity chromatography

Seralose-galactose gel was packed into a glass column and equilibrated with 0.15 M NaCl. The protein sample obtained above was then applied on the gel. The gel was washed extensively to remove unbound proteins and the bound protein eluted specifically using 0.3 M galactose in 0.15 M NaCl. Protein was monitored at 280 nm. Protein containing fractions were dialyzed and tested for hemagglutinating activity using pronase treated rabbit erythrocytes. The active fractions were pooled, concentrated and subjected to gel filtration.

3.2.7. Molecular mass determination

The fractions containing the lectin obtained above were pooled, concentrated and applied on to a Sephadex G-150 gel (75 cm x 1.3 cm) equilibrated with 0.15 mM NaCl at a flow rate of 20 mL/h. The column was calibrated with proteins of known molecular weight viz., BSA (66 kDa), ovalbumin (45 kDa) and lysozyme (14.7 kDa) respectively. MoSL eluted as a single peak and its native molecular mass was determined using the standard graph.

3.2.8. Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 5% stacking gel and 10% resolving gel according to Laemmli [1970] under non-reducing and reducing conditions. After the electrophoretic run, the gels were stained with coomassie brilliant blue R-250. At low protein concentration, the gels were silver stained. The standard protein marker used is medium range Fermentas marker consisting of β- galactosidase (116.0 kDa), BSA

(66.2 kDa), ovalbumin (45.0 kDa), lactate degyhydrogenase (35.0 kDa), REase Bsp 981 (25.0 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa).

3.2.9. Protein concentration

The protein content of the lectin samples obtained during the purification process was determined by the dye-binding method of Bradford with bovine serum albumin (1 mg/mL) as the standard [Bradford, 1976]. Protein in column fractions was monitored by measuring the absorbance at 280 nm.

3.2.10. Carbohydrate content

The neutral sugar content of MoSL was estimated by the phenol–sulfuric acid method using glucose as the standard [Dubois *et al.*, 1956].

3.2.11. Sugar inhibition studies

The assay with the purified lectin was performed as follows: 50 µL of different sugar solutions (0.4 M) (10 mM for synthetic sugars) were placed in the plexi plate and serially diluted. Then, 50 µL of the purified lectin (75 µg) was added to each well. The reaction mixture was incubated for 30 min at 37°C. To this pronase treated rabbit erythrocytes were added as described above and the agglutination visualized and titers noted.

3.2.12. Effect of pH, temperature and EDTA

To see the effect of pH on the lectin activity, 150 µg of MoSL was incubated for 6 h at various pH buffers ranging from pH 2.0 to 8.0, residual lectin activity was tested using standard hemagglutination assay. To investigate the thermostability, 1 mg/ mL concentration of the lectin was incubated at different temperatures of 4, 30, 40, 50, 60, 70, 80, and 90°C for a period of 30 min. The samples were brought back to room temperature and their ability to agglutinate the pronase treated rabbit erythrocytes were tested according to the method described above. To test the requirement of divalent cations for lectin activity, the sample was dialyzed against 100 mM EDTA in 100 mM sodium acetate buffer, pH 6.0 for 24 h. The solution was then dialyzed against distilled water for 24 h to remove EDTA. The lectin solution was then tested for hemagglutinating activity in the absence or presence of 10 mM $CaCl_2$, $MnCl_2$, or $MgCl_2$ in 0.15 M NaCl.

3.2.13. Lectin Affi-gel preparation

Coupling of the purified MoSL to Affigel-10 was carried out following manufacturer's instructions. Purified MoSL was dialyzed against 0.1 M HEPES buffer pH 7.4. 2.0 mL of Affigel-10 (Bio-rad labs) was thoroughly washed with chilled isoproponal followed by cold-water and 0.1 M HEPES buffer pH 7.4. To this 10 mg/mL MoSL in HEPES buffer, was added and the coupling reaction allowed to proceed at 4°C for 24 h by end over end rotation. At the end of this incubation period, the unbound fraction collected to determine the extent of binding and the unreacted sites in the gel were blocked with 0.1 M-ethanolamine-HCl pH 8.0 (200 µL/mL Affigel-10) for 1 h at 4°C. The gel was finally washed with PBS and equilibrated with sodium acetate buffer pH 5.0 for further experimentation.

3.2.14. Immobilized lectin-affinity chromatography

Lectin affigel prepared above was used in this study. The unbound fraction from the Seralose-galactose gel (described in methods section 3.2.6), devoid of lectin activity was dialyzed against 50 mM sodium acetate buffer pH 5.0 and analyzed for various glycosidase activities. Significantly α-D-galactosidase, α-D-mannosidase, β-D-glucosidase and α-D-glucosidase were detectable (Assay described in chapter 4A). This sample was passed through the lectin-affigel which was previously equilibrated with pH 5.0 buffer and the gel extensively washed with the same buffer until the A_{280} of the unbound fractions was <0.05. The bound proteins were eluted using 25 mM Tris-HCl buffer pH 8.0. Aliquots of the eluted fractions were analyzed by SDS-PAGE to know the nature of protein bands.

3.2.15. Proteomic analyses: in-gel digestion and mass spectrometry (MS)

After SDS-PAGE of the purified lectin, the protein was stained with CBB for detection of the lectin band. The excised gel pieces were destained with 100 µL of 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate (NH_4HCO_3) for five times. In-gel digestion and matrix-assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF MS) analysis was conducted with a MALDI-TOF/TOF mass spectrometer (Bruker Autoflex III smartbeam, Bruker Daltonics, Bremen, Germany) according to the method described by Shevchenko *et al.* [1996] with slight modifications. The gel pieces were treated with 10 mM DTT in 25 mM NH_4HCO_3 and incubated at 56°C for 1 h. This is followed by treatment with 55 mM

Iodoacetamide in 25 mM NH_4HCO_3 for 45 min at room temperature (25 ± 2°C), washed with 25 mM NH_4HCO_3 and ACN, dried in speed vac and rehydrated in 20 µL of 25 mM NH_4HCO_3 solution containing 12.5 ng μL^{-1} trypsin (sequencing grade, Promega, Wisconsin, USA). The above mixture was incubated on ice for 10 min and kept overnight for digestion at 37°C. After digestion, a short spin for 10 min was given and the supernatant was collected in a fresh eppendorf tube. The gel pieces were re-extracted with 50 µL of 1% trifluoroacetic acid (TFA) and ACN (1:1) for 15 min with frequent vortexing. The supernatants were pooled together and dried using speed vac and were reconstituted in 5 µL of 1:1 ACN and 1% TFA. 2 µL of the above sample was mixed with 2 µL of freshly prepared α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN and 1% TFA (1:1) and 1 µL was spotted on target plate.

Protein identification: peptide mass fingerprinting and MS/MS analysis.

Protein identification was performed by database searches (PMF and MS/MS) using MASCOT program (http://www.matrixscience.com) employing Biotools software (Bruker Daltonics).The similarity search for mass values was done with existing digests and sequence information from NCBInr and Swiss Prot database. The taxonomic category was set to Viridiplantae (green plants). The other search parameters were: fixed modification of carbamidomethyl (C), variable modification of oxidation (M), enzyme trypsin, peptide charge of 1^+ and monoisotropic. According to the MASCOT probability analysis ($P<0.05$), only significant hits were accepted for protein identification.

3.2.16. Serological cross reactivity

Ouchterlony's double immunodiffusion was performed to study serological cross-reactions with other galactose specific lectins. Immunodiffusion was carried out on 1% agar (in PBS) plates for 24-48 hours at 37°C in a humidified chamber and visualised for precipitin arcs [Ouchterlony, 1948]. Antiserum was placed in the central well and the lectin samples were placed in the other wells.

3.3.0. Results

3.3.1. Extraction and purification of MoSL

Seeds from *M.oleifera* contain a galactose binding lectin (MoSL) that was isolated by affinity chromatography, followed by gel permeation chromatography. The purification of the lectin from the seed extract is summarized [Table: 3.1]. The crude extract of the *M.oleifera* seeds after ammonium sulphate precipitation did not show any agglutination activity with untreated and trypsin treated rabbit erythrocytes. However, agglutination with titer value of 2^7 was observed with the pronase treated rabbit erythrocytes [Table: 3.2]. With the initial results of the sugar inhibition with the crude extract, galactose showed pronounced inhibition (MIC: 4mM) followed by lactose (MIC: 15 mM). Using this information we prepared a Seralose-galactose gel for the affinity purification of this lectin. MoSL was strongly bound to the gel and specifically eluted as a single peak from the Seralose-galactose gel using 0.3 M galactose in the column buffer [Figure: 3.1]. To further analyze the homogeneity of the purified lectin gel filtration was used. The same gel filtration column after calibrating with known standard protein is used to determine the native molecular mass of the MoSL. The protein was eluted as a single major protein peak which also exhibited the lectin activity [Figure: 3.2 A & B]. The purified lectin migrated as a single band corresponds to 27.0 kDa in SDS-PAGE under both reducing and non-reducing conditions [Figure: 3.3]. The results of SDS-PAGE and gel filtration indicated that this lectin is a monomer with an apparent molecular mass of 27 kDa. The calculated pI value of the MoSL lectin was 8.6, as suggested by MALDI report. Phenol sulphuric acid method did not show detectable range of carbohydrate indicating that this is not a glycoprotein.

3.3.2. Sugar inhibition

The influence of carbohydrates on hemagglutination activity of MoSL was studied by incubating the lectin with various simple and synthetic sugars [structures shown in Figure: 3.4] as described under methods. Agglutination mediated through MoSL has been studied in absence and in the presence of the synthetic glycoconjugates. Simple carbohydrates, viz., Mannose and Glucose don't show inhibition of agglutination activity up to a concentration of 200 mM and 400 mM respectively. Even the conjugates, viz., Glucosyl Naphthyl Imine (Glu2NI), Glucosyl Salicilyl Imine

(Glu2SI) and Mannosyl Naphthyl Imine (MNI), showed no inhibition until a concentration of 10 mM respectively. On the other hand, galactose and its conjugates, viz., Galactose (Gal), Galactosyl C1-Amine (GC1A), Galactosyl Salicilyl Imine (GSI) and Galactosyl Naphthyl Imine (GNI), were found to inhibit the agglutination even at a minimum concentration of 3.12, 1.56, 0.39, 0.04 mM respectively. Thus the inhibition of agglutination among the galactosyl based ones follow a trend, viz., GNI > GSI > GC1A > Gal [Table: 3.3].

3.3.3. Effect of temperature, pH and EDTA on hemagglutination activity

The lectin activity is stable from pH 4.0 to 6.0 [Figure: 3.5-A]. The effect of temperature on the hemagglutinating activity of MoSL is shown in Figure: 3.5-B. It is evident that MoSL was relatively stable below 70°C. However, the hemagglutinating activity of MoSL declined markedly at 80°C, and the lectin was completely inactive at 90°C. MoSL showed no change in its hemagglutinating activity after treatment with EDTA, suggesting that MoSL doesn't require metal ion for its activity.

3.3.4. Lectin affigel

Many plant lectins have been studied for their interactions with glycosidases. Immobilized lectin-affinity chromatography using MoSL affigel showed that there is some interaction between MoSL and glycosidases which are present in the same seeds [Figure: 3.6 A & B]

3.3.5. Denovo peptide sequencing

Peptide mass fingerprinting of the internal peptide sequence of the purified MoSL resulted after digestion with the trypsin was searched against the plant kingdom using Mascot search. MS/MS data obtained from this does not share similarities with any know lectin studied so far [Figure: 3.7]

3.3.6. Cross-reactivity of the MoSL with the anti-DLL antibodies

The degree of immunological homology between MoSL and a galactose specific lectin from *Dolichos lablab* (DLL-II) which was purified and characterized in our laboratory was assessed using double-immunodiffusion experiments, the lectin reacted with the antiserum to the galactose specific lectin DLL-II giving a single precipitin line, indicating that the antiserum specifically recognises the MoSL [Figure:

3.8-A]. Pre-immune serum did not give any precipitin line. This is further confirmed in western blot developed with DLL-II antibody [Figure: 3.8-B].

FIGURES

Figure: 3.1.

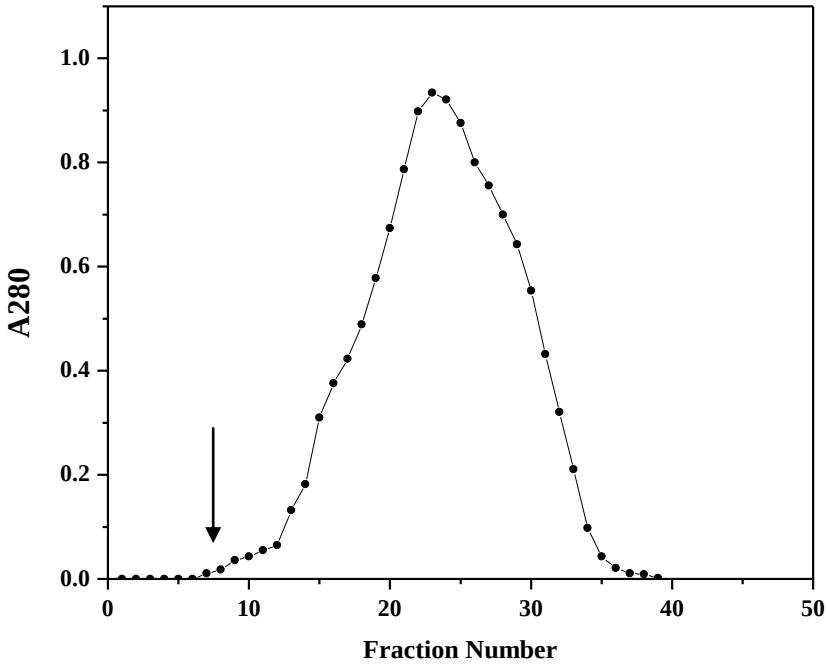
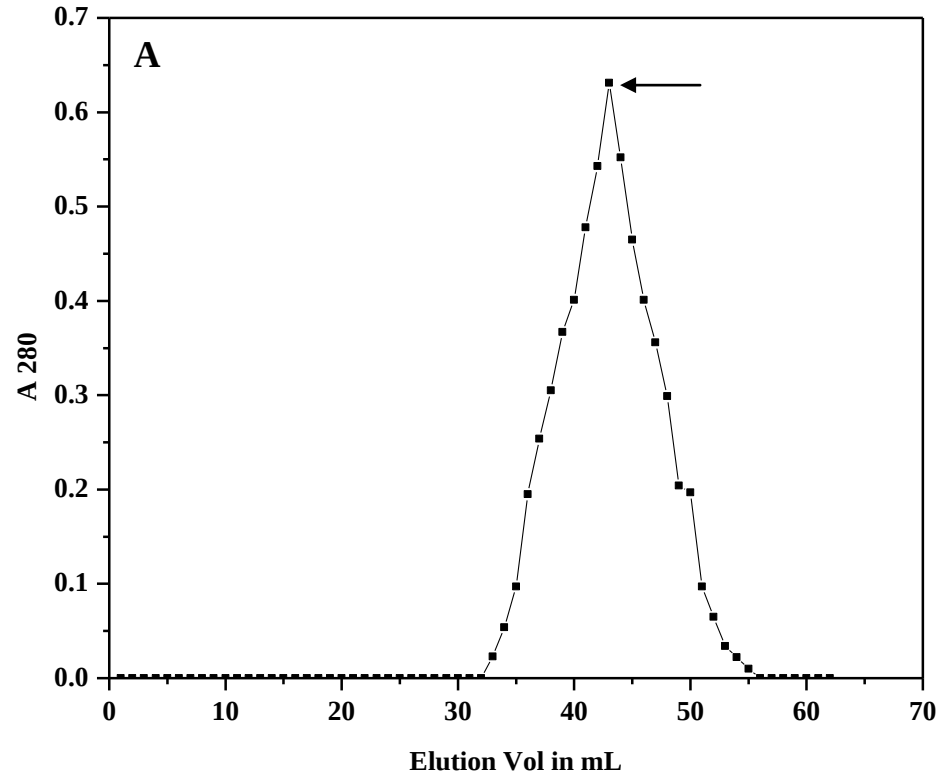


Figure: 3.1. Elution profile of MoSL from the affinity gel (3 x 15 cm): The ammonium sulphate fraction (70%) of *Moringa oleifera* saline extract was loaded on to the Seralose-galactose gel which was previously equilibrated with saline and washed to remove unbound proteins. The column was eluted with 0.3 M galactose in 0.15 M NaCl and fractions of 1 mL were collected manually. Protein was monitored at 280 nm and haemagglutinating activity checked after dialyzing the sample. Arrow indicates point where 0.3 M galactose was applied.

Figure: 3.2 A & B.


A
A 280
Elution Vol in mL
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
10
20
30
40
50
60
70

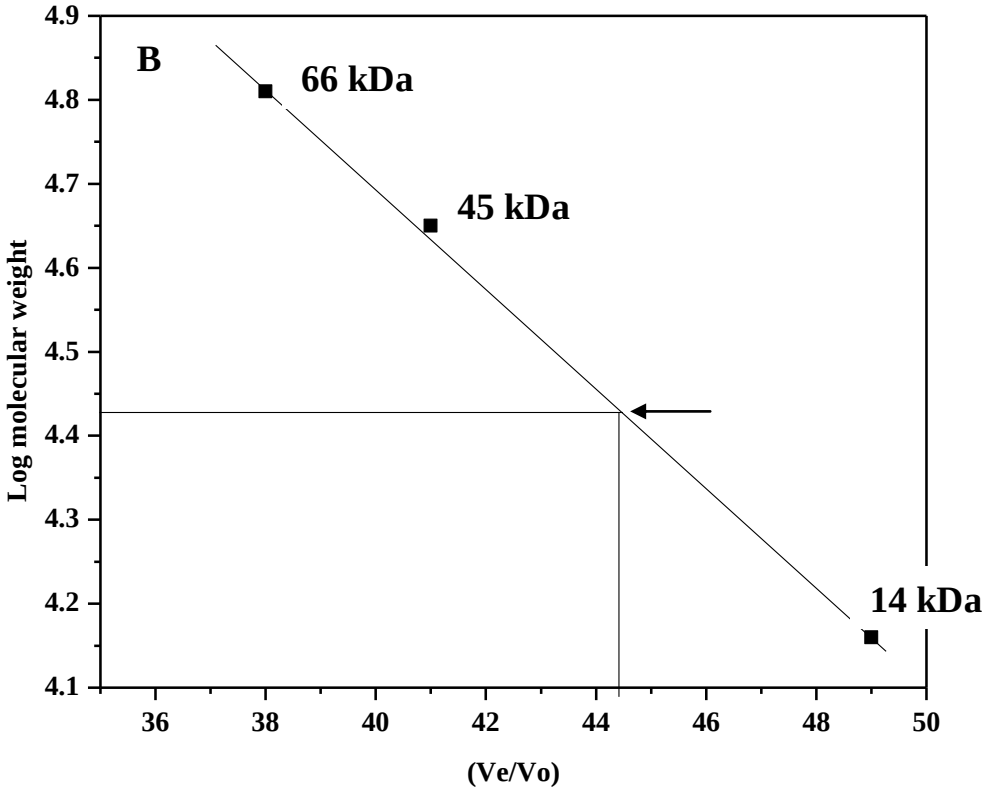

B
66 kDa
45 kDa
14 kDa
Log molecular weight
(Ve/Vo)
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
36
38
40
42
44
46
48
50

Figure: 3.2 A & B. Gel filtration and molecular weight determination of the purified lectin using G-150 column (75 x 1.3 cm): (A) The galactose gel eluted lectin sample was concentrated and passed through Sephadex G-150 column. Both equilibration and elution was carried with 0.15 M NaCl. Arrow indicates peak point where the lectin was eluted. **(B)** The same column was calibrated with known standard proteins i.e. BSA (66.0 kDa), Ovalbumin (45.0 kDa), Lysozyme (14.7 kDa) respectively. Arrow indicates fraction at which purified sample (Mr 27.0 kDa) was eluted.

Figure: 3.3

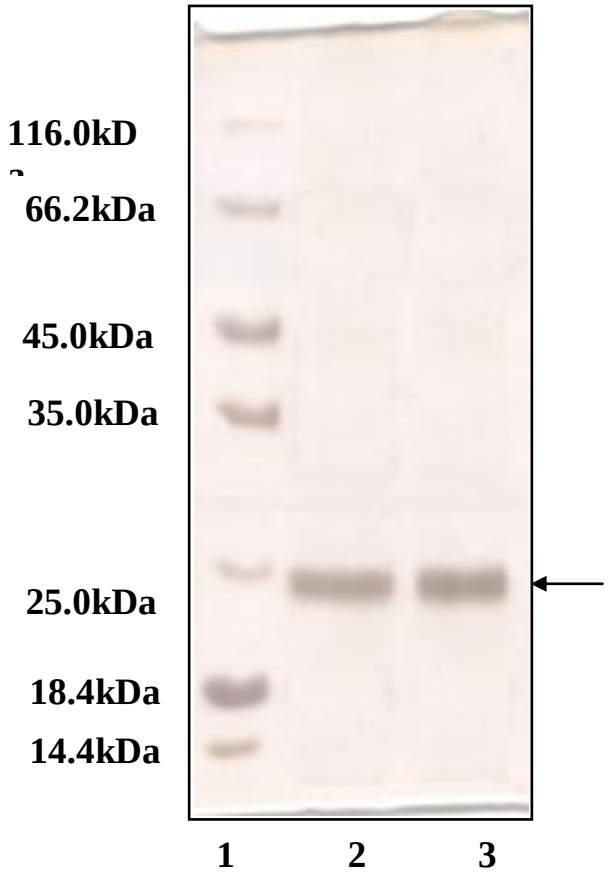
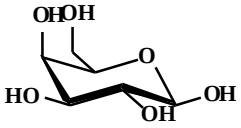


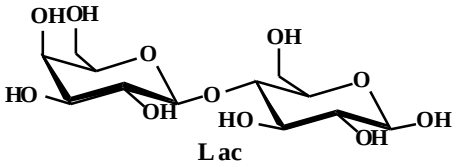
Figure: 3.3. 12.5 % Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of *Moringa oleifera* Seed Lectin (MoSL). Lane 1: - Medium range molecular weight marker. Lane 2 & 3: - *Moringa oleifera* Seed Lectin after gel filtration under reducing and non-reducing conditions. The arrow corresponds to purified lectin (Silver staining). The standard protein markers used are medium range Fermentas markers consisting of β- galactosidase (116.0 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), lactate degyhydrogenase (35.0 kDa), REase Bsp 981 (25 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa).

Figure: 3.4.



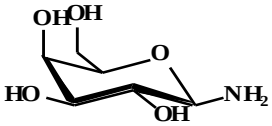
Gal

Galactose



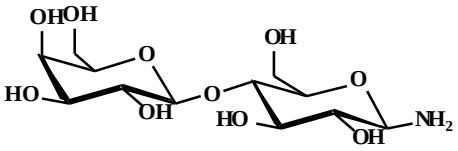
Lac

Lactose



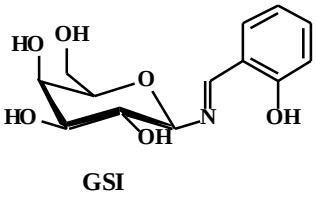
GC1A

Galactosyl C1 – Amine



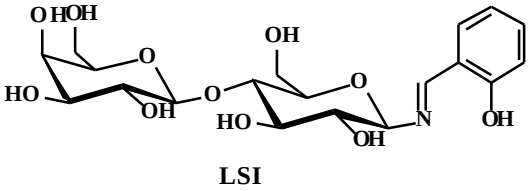
LC1A

Lactosyl C1 – Amine



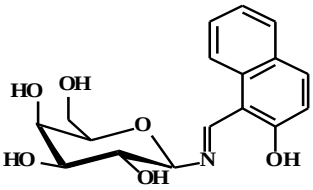
GSI

Galactosyl Salicilyl Imine



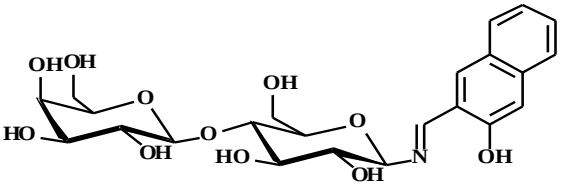
LSI

Lactose Salicilyl Imine



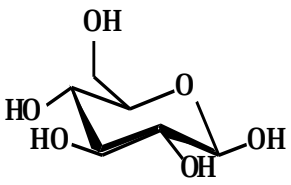
GNI

Galactosyl Naphthyl Imine



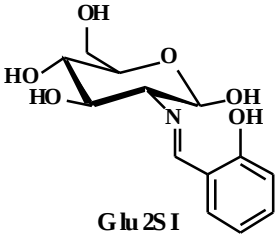
LNI

Lactose Naphthyl Imine



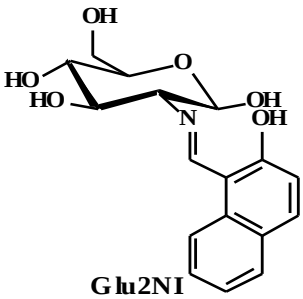
Glu

Glucose



Glu2SI

Glucosyl Salicilyl Imine

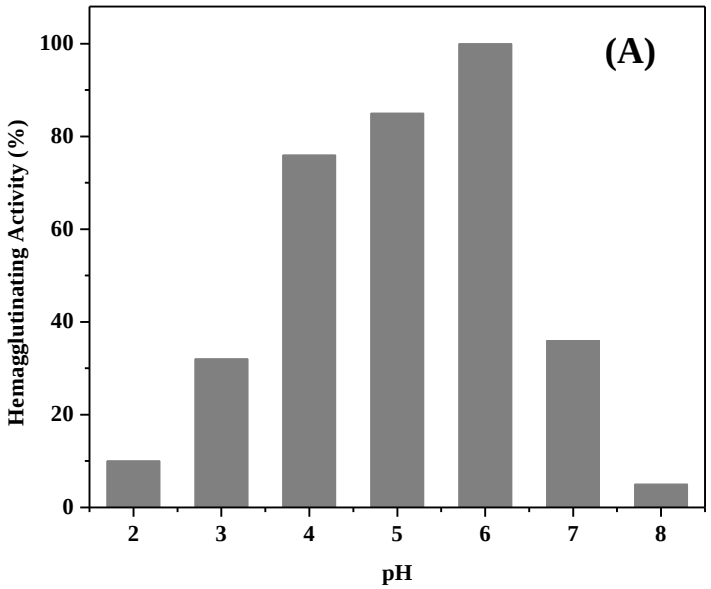


Glu2NI

Glucosyl Naphthyl Imine

Figure: 3.4. Synthetic glyco-conjugates used in the present study: Synthesis and characterization of glyco-conjugates are detailed in Kumar *et al.* (2009). These were kindly provided by Prof. C. P. Rao, IIT Mumbai.

Figure: 3.5 A & B


(A)
Hemagglutinating Activity (%)
0
20
40
60
80
100
2
3
4
5
6
7
8
pH

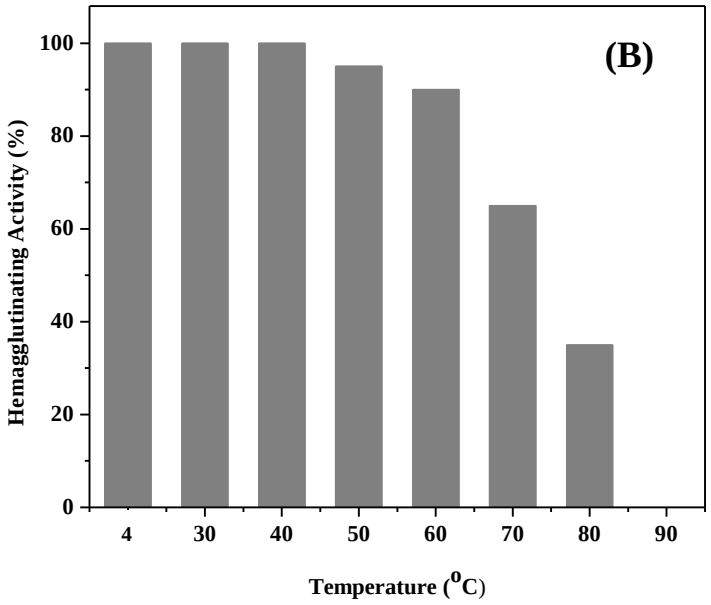

(B)
Hemagglutinating Activity (%)
0
20
40
60
80
100
4
30
40
50
60
70
80
90
Temperature (°C)

Figure: 3.5 A & B. (A) Effect of pH and (B) Temperature on the MoSL: The effect of pH and Temperature on MoSL activity was carried out at various pH and temperatures as described in the materials and methods. Same amount of protein was used at different pH values and at pH 6.0 the activity is taken as 100%.

Figure: 3.6 A & B

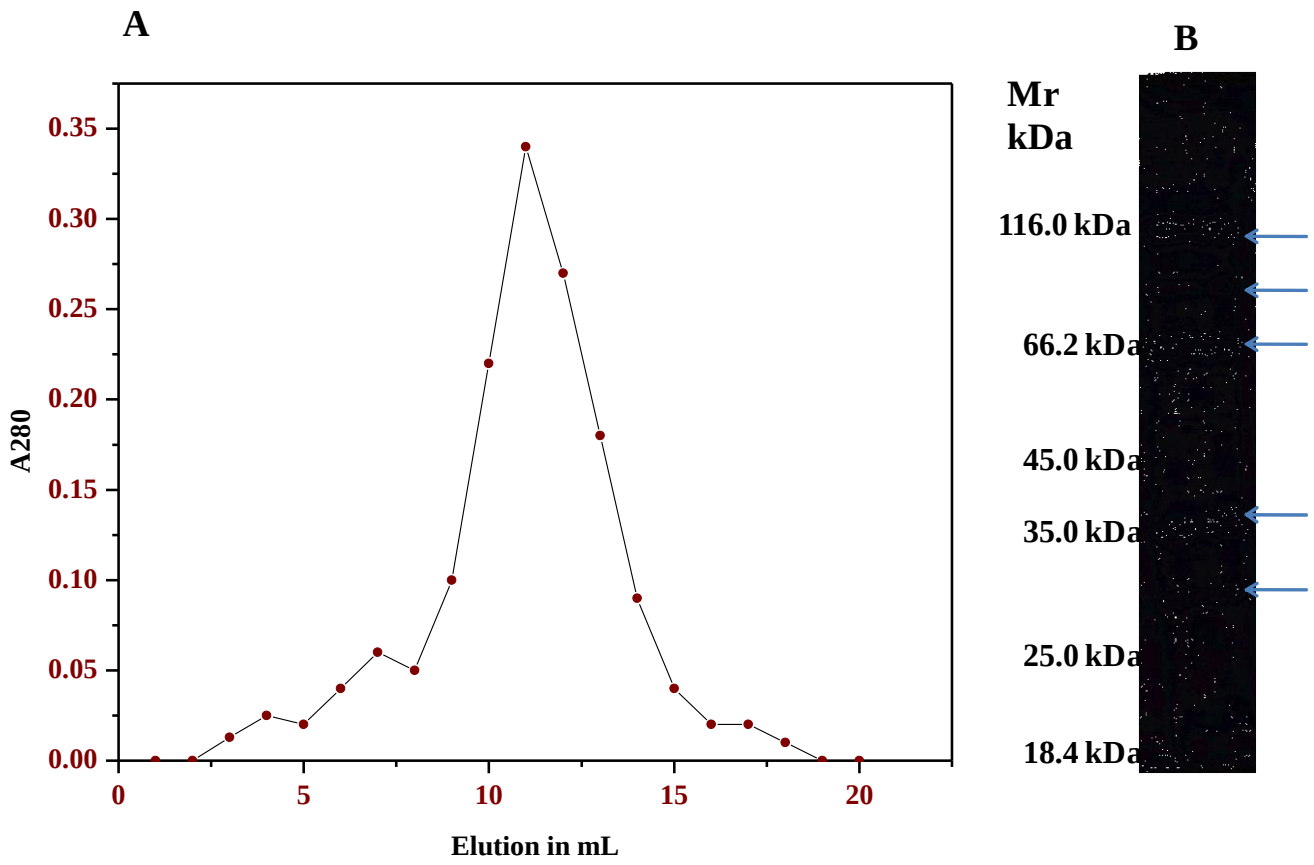

A
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A280
0
5
10
15
20
Elution in mL
B
Mr
kDa
116.0 kDa
66.2 kDa
45.0 kDa
35.0 kDa
25.0 kDa
18.4 kDa

Figure: 3.6 A & B. Immobilized lectin affinity chromatography: (A) Glycosidase rich crude seed extract in 20 mM sodium acetate buffer pH 5.0 containing 150 mM NaCl was loaded. The column was washed with equilibrating buffer until A_{280} was zero. Bound proteins were eluted with 20 mM Tris-Hcl buffer pH 8.0 containing 150 mM NaCl. As shown in the Figure A. **(B) SDS–PAGE of the eluted proteins**. Electrophoresis was carried out in a 10% gel as described in the Materials and methods. The arrows on the right indicates the proteins eluted from the gel, that also showed glycosidase activities.

Figure: 3.7

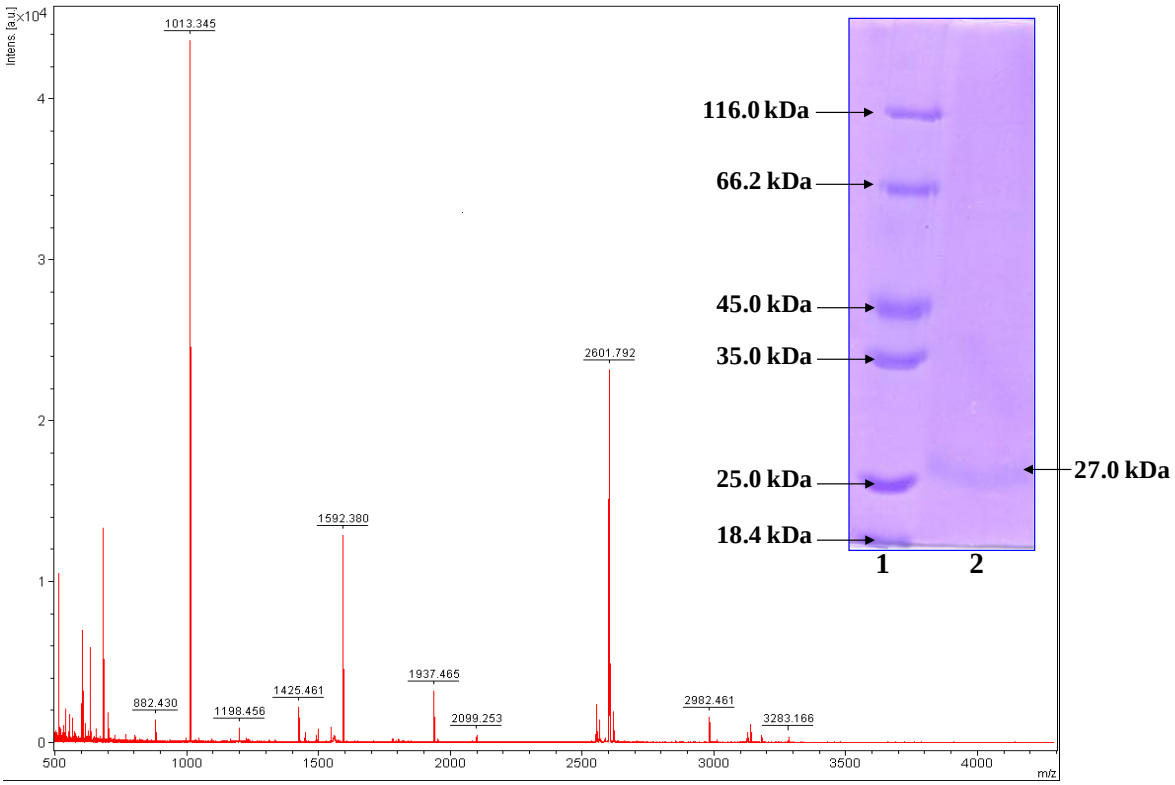
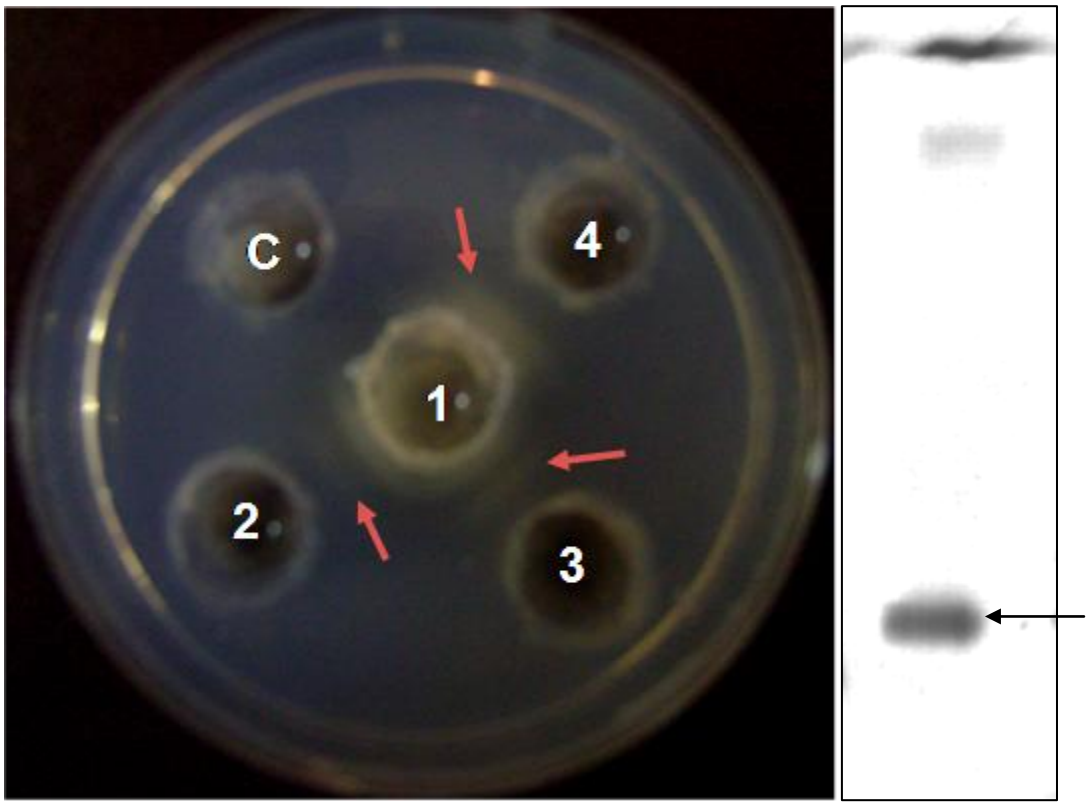

Intens. [a.u.]
×10^4
1013.345
2601.792
1592.380
1937.465
1425.461
882.430
1198.456
2099.253
2982.461
3283.166
m/z
116.0 kDa
66.2 kDa
45.0 kDa
35.0 kDa
25.0 kDa
18.4 kDa
27.0 kDa
1
2

Figure: 3.7. Internal peptide analysis of MoSL by MALDI-TOF. MALDI-TOF analysis was performed with proteolytic digests of the MoSL using trypsin. Displayed are the peptide mass fingerprints peaks. Inset 12.5% SDS-PAGE of the purified MoSL stained with Coomassie R250. Arrow represents the lectin band which is excised and given for the MALDI analysis.

Figure: 3.8 A & B



A **B**

Figure: 3.8 A & B. Serological cross-reactivity: (A) Immunodiffusion of MoSL with that of *Dolichos lablab* seed lectin antibody: Well 1: DLL-II (Galactose specific lectin antibody available in lab), Well 2: Crude extraction of *Moringa oleifera* seeds, Well 3: Purified MoSL, Well 4: DLL-II (positive control) and Well C: PBS buffer (negative control). Arrows indicates precipitin line (Arc). **(B)** MoSL lectin blot using DLL-II antiserum as the primary antibody. Arrow indicates the lectin band in the blot developed with BCIP/NBT.

TABLES

Table: 3.1. Purification of *Moringa oleifera* Seed Lectin (MoSL)

Fraction	Total protein Volume(mL)	Total protein (mg)	Total Activity (HU) [b]	Specific Activity (HU/mg)	Purification fold	Yield (%)
Saline extract [a]	500	800	1000	1.25	1	100
Galactose-Sepharose eluate	30	40	865	21	16.8	86.5
G-150 Gel filtration	22	33	730	22.12	17.6	73

[a] 100 g of the seeds were used.

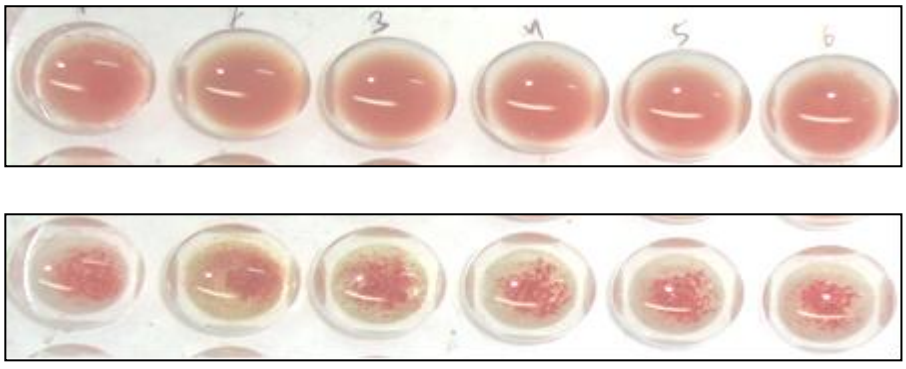
[b] Hemagglutination unit (HU) is defined as the minimal concentration of protein required to cause visible agglutination of a 4% pronase treated rabbit erythrocytes.

Table: 3.2. Hemagglutinating activity of *Moringa oleifera* seed lectin (MoSL) against native, trypsin and pronase treated rabbit erythrocytes.

Source	Type	Agglutination (titer)
Rabbit	**Native**	**ND**
	Trypsin treated (1%)	**ND**
	Pronase treated (0.05%)	**2^7**

2^7 denotes the dilution factor causing visible agglutination of pronase treated rabbit erythrocytes.

ND: Not Detected



Control

Agglutination pattern

Table: 3.3. Influence of simple and synthetic sugars on the purified MoSL.

Simple Sugar	MIC (mM)	Synthetic Sugar	MIC (mM)
D-glucose	NI	Galactosyl C_1 – Amine	1.56
D-mannose	200	Lactosyl C_1 – Amine	1.56
D-galactose	3.12	Galactosyl Salicilyl Imine	0.39
D-fucose	4.1	Galactosyl Naphthyl Imine	0.04
Lactose	12.5	Mannosyl Naphthyl Imine	NI
Melibiose	12.5	Lactose Salicilyl Imine	1.56
Arabinose	NI	Lactose Naphthyl Imine	1.1
L-rhamnose	9.8	Glucosyl Salicilyl Imine	NI
Stachyose	8.2	Glucosyl Naphthyl Imine	NI

MIC: Minimum Inhibitory Concentration,

NI: No Inhibition at 400 mM Concentration for simple sugars and 10 mM for synthetic sugars.

3.4.0. Discussion

The *M.oleifera* occurs widely in the tropical and subtropical regions of India. In the present study, we isolated and characterized a new D-galactose-binding lectin, MoSL from the seeds of *M.oleifera*. Though lectins were identified from *M.oleifera* seeds by other researchers, the lectin we isolated exhibits properties distinct from those reported [Santos *et al.,* 2005; Katre *et al.,* 2008]. The agglutination activity was observed in saline extracted samples. Hemagglutination activity test showed that MoSL was able to agglutinate only pronase treated rabbit erythrocytes and the hemagglutinating activity could be inhibited by galactose and its derivatives. This nature of agglutination activity may be due to the nature of the glycoproteins protruding on the cell surface, which are weakly or not totally recognized by the lectin. Iglesisa *et al.,* [1982] reported that purified lectin from *Erythrina cristagalli* did not agglutinate native mouse erythrocytes upto a concentration of 1 mg/mL. These properties of the lectin were also seen in *Cicer arietinum* (chickpea) lectin which showed agglutination only with the pronase-treated rabbit or human erythrocytes [Katre *et al.,* 2005]. WSMoL showed agglutination with glutaraldehyde-treated rabbit erythrocytes [Santos *et al.,* 2005]. Thus different lectins seem to show distinct agglutination patterns with different animal erythrocytes.

The Seralose-galactose gel used to affinity purify the MoSL yields the lectin in a pure form and the fact that the lectin can be eluted using 0.3 M galactose signifies its sugar specificity. From 100 g of *Moringa* seeds 33 mg of purified MoSL were obtained [Table: 3.1] (lectin was purified in batch wise). Seralose-galactose gel has earlier been tested in our laboratory for the purification of the *Dolichos lablab* galactose specific lectin (DLL-II) [Latha *et al.,* 2006; Rao and Nadimpalli, 2007].

Gel filtration experiments showed that MoSL was approximately 27 kDa monomeric and non-glycosylated protein. However, the molecular weight of WSMoL and MoL reported in literature were 20 kDa and 14 kDa respectively. The non glycoprotein nature is common in some lectins. cMOL is also a non-glycoprotein hemagglutinin where as WSMoL is a glycopolypeptide. The hemagglutination activity of MoSL was inhibited by D-galactose and its derivatives. This inhibitory property is similar to that of other D-galactose-binding lectins [Yan *et al.,* 2005]. Katre *et al.,* [2008] isolated a *Moringa oleifera* seed hemagglutinin (MoL) whose activity was inhibited by complex

carbohydrates like thyroglobulin, fetuin and holotransferin. In this respect the lectin we isolated appears different in its properties compared to already published reports.

Different glycoses were modified either at their C1- (Gal and Lac) or at their C2- (Glc) positions to result in glycoconjugates possessing amino (GC1A and LC1A) and aromatic-imino moieties (GSI, GNI, LSI, LNI, Glu2SI and Glu2NI) as shown in Figure: 3.4 by adopting the synthetic methodologies described by Kumar *et al.* [2009]. All the aromatic-glyco-imino-conjugates and appropriate controls have been used in the present study. It is interesting to note that napthyl imine derivatives of galactose and lactose showed 10 times more inhibition than Salicilyl imine derivatives of the same sugars, whereas, 79 times more than the simple galactose. These results suggest that the napthyl imine at C1 position posses more inhibitory effect by readily binding to the active sites of the lectin and preventing it from exhibiting hemagglutination. The better inhibition of synthetic sugars over the galactose indicated that MoSL may possess a hydrophobic –binding pocket in the proximity to the lectin sugar affinity site. Synthetic sugars which are derived from the natural sugars showed greater inhibition. Similar results were also observed for the galactose specific jacalin lectin [Kumar *et al.,* 2010]. Availability of large concentrations of the synthetic sugars should be very useful for large scale isolation of the MoSL. The biological activities of this lectin shown here highlight the importance of sugar–protein interactions which can possibly have applications in biological research.

The optimal pH of the lectin was slightly in the acidic range as has been seen for WSMoL [Santos *et al.,* 2005]. The optimal pH of WSMoL is around pH 4.5 and agglutinating property is completely abolished at pH 7.0. Whereas, MoSL activity is not abolished at pH 7.0. The lectin exhibited thermo stability until 70°C and is similar to other lectin isolated from this plant [Katre *et al.,* 2008; Santos *et al.,* 2009]

The MS/MS data generated from the peptide mass fingerprinting of the purified MoSL did not show any similarity with any known lectin. This implies that this is a new type of lectin. However, it is necessary to obtain complete sequence information of MoSL to support this statement and to correlate with other lectins.

The degree of immunological relatedness between MoSL and DLL-II was assessed by Ouchterlony's double diffusion method [Ouchterlony, 1948]. Figure 3.7 show the typical results for the study. The antibodies raised against DLL-II a known galactose-

specific lectin, formed a single precipitin line with MoSL. These results are interesting that lectins from different families that have similar sugar specificity share unique immunological relatedness. Many legume lectins show high degree of sequence identity and immunological relatedness [Rao and Nadimpalli, 2007].

Lectins are thought to be involved in plant defense system by being resistant against insects, bacteria and fungi. In particular chitin binding lectins seems to have a role in defending plant against insects. Some of the proven insecticidal lectins are GNA (*Galanthus nivalis* agglutinin, GNA) ConA (Mannose/glucose specifies), PNA (galactose specifies), wheat germ agglutinin (WGA) [Macedo *et al.,* 2002]. Recent finding showed that cMoL a seed lectin of *M.oleifera* showed insecticidal activity on the survival and growth of the Mediterranean flour moth, Anagasta kuehniella (Zeller) (Lepidoptera: Pyralidae) [Oliveira *et al.,* 2010]. Another lectin from *M.oleifera*, WSMoL (**W**ater **S**oluble ***M****oringa* ***o****leifera* **L**ectin), showed larvicidal activity to *Aedes aegypti*, the vector of dengue [Coelho *et al.,* 2009].

Furthermore, Water soluble lectin (WSMoL) from this source is mainly observed in the seed coat, it is released upon seed imbibition at pH 6.0. This property of releasing the lectin into the buffer upon imbibition suggests the protective function in the early stage of germination when the seeds are particularly vulnerable to pathogen attacks. It would be interesting to carryout studies with the purified MoSL to analyze its insecticidal activity and to understand its specific *in vivo* role in causing protection against any pathogens.

In the present study, we have isolated and extensively characterized a galactose-binding lectin from the seeds of *M.oleifera*. Though MoSL shares some degree of similarity with other lectins isolated from the same source in terms of molecular weight and physicochemical parameters, it showed distinct variations from other members in its carbohydrate binding ability. It is interesting from a comparative biochemical point of view that lectins with different sugar-binding specificity occur in the same plant. It is noteworthy that homologous lectins are expressed in several different tissues of the same plant. In addition, MoSL showed considerably different characteristics including molecular mass and sugar-recognition specificity, and we conclude that at least, four different kinds of lectins (WSMoL, MoL, cMoL and MoSL) are present in *M.oleifera* seeds. These differences may be attributed to the variation in the variety of seeds used in each study, there origin and storage

conditions. To obtain a clear information about the relation between these lectins a complete primary sequencing should be carried out which is beyond the scope of present investigation. Our studies reveal that the *Moringa oleifera* seed lectin (MoSL) we isolated and characterized is a novel lectin with potent and potentially exploitable activities.

CHAPTER 4

Glycosidases from the *Moringa oleifera* seeds

4A. Purification and characterization of α-mannosidase (EC 3.2.1.24) from *Moringa oleifera* and the effect of glyco-conjugates on its activity.

4B. Purification, Biochemical characterization and localization studies of acidic α-galactosidase from *Moringa oleifera* seeds.

CHAPTER 4A

Purification and characterization of α-mannosidase (EC 3.2.1.24) from *Moringa oleifera* and the effect of glyco-conjugates on its activity

4A.1.0. Introduction

The enzyme α-mannosidases (E.C.3.2.1.24: α-D-mannosidase) are widely distributed in animals, plants and microorganisms [Kornfeld and Kornfeld, 1985]. They play a key role in biosynthesis and the turnover of *N*-linked glycoproteins which are involved in important biological activities in the cells [Hossain *et al.,* 2009]. To date, several α-mannosidases have been purified and characterized from different legumes [Li, 1967; Paus, 1977; Tulasi and Nadimpalli, 1997] and non legume sources [Ohtani and Misaki, 1983; Priya Sethu and Prabha 1997; Kishimoto *et al.,* 2001; Mahadevi *et al.,* 2002]. This enzyme is an abundant constituent of the plant hydrolytic system [Snaith, 1975]. α-mannosidases can be classified into two types: one is involved in the biosynthesis (or processing) of *N*-linked glycoproteins [Lerouge, 1998], the other in the degradation of *N*-glycans [Kimura *et al.,* 1999; Kishimoto *et al.,* 2001].The plant α-mannosidase that has been extensively studied is from the seeds of *Canavalia ensiformis* (jack bean) [Snaith, 1975; Howard *et al.,* 1998; Einhoff and Rudiger, 1998]. The Jack bean α-mannosidase was isolated by chromatography on concanavalin A, the lectin from the same plant, without involving its sugar binding site [Einhoff and Rudiger, 1986]. Jack bean α-mannosidase is a tetrameric protein, with two different subunits (66 kDa and 44 kDa), in which the larger subunit is glycosylated and it is zinc containing metalloprotein [Kimura *et al.,* 1999]. Furthermore, the formation of oligomeric structure with all the subunits present was found to be essential for the enzyme activity that is involved in the degradation of the *N*-glycoproteins. The α-mannosidases have been classified into two independently derived groups, Class I and Class II, based on the biochemical properties, substrate specificity, inhibitor profiles, catalytic mechanism and characteristic regions of conserved amino acid sequences and sequence alignments. In plant system this is based on the inhibitor profiles alone which characterize these groups [Daniel *et al.,* 1994; Moremen *et al.,* 1994; Eades *et al.,* 1998]. Jack Bean α-Mannosidases analogous to human lysosomal α-mannosidase are retaining glycosidases which are inhibited by Swainsonine a potent α-mannosidase II inhibitor [Howard *et al.,* 1997].

Plant α-mannosidases generally require metal ions for their activity, though zinc was found to be more common divalent metal ion required for their activities, other metal ions such as Co (II), Cd (II) were also found to enhance the enzyme activity [Nakajima *et al.,* 2003; Woo *et al.,* 2004]. The metal can be chelated using EDTA with loss of enzyme activity. The activity is regained by supplementing zinc externally [Snaith, 1975]. The involvement of the tryptophan residues at the active site was previously demonstrated in *Phaseolus vulgaris* (pinto beans) and Jack bean α-mannosidase [Paus, 1978; Burrows and Rastall, 1998]. It has been shown that the levels of the α-mannosidase increase during seed germination and fruit ripening suggesting its role in removing mannose residues from mannoglycans from the cell wall glycans [Kestwal *et al.,* 2007]. The enzyme has been used for the structural elucidation of glycoproteins and glycolipids. In particular, the α-mannosidase from jack bean whose properties have been extensively characterized has generally been employed in determining the glycan structure [Misaki *et al.,* 2003]. Furthermore, studies were carried out for designing potential glycosidase inhibitors derived from simple carbohydrates. It gives important information regarding the involvement of aromatic, imine and carbohydrate moieties of these inhibitors in effective inhibition [Kumar *et al.,* 2009].

Previously in our laboratory, we have purified and characterized α-mannosidase both from legume and non-legume sources (lablab beans and Triticale). This hydrolytic enzyme along with lectins is localized in protein bodies [Tulasi and Nadimpalli, 1997; Mahadevi *et al.,* 2002].

During the course of the initial studies on some biologically active proteins from *Moringa oleifera*, a multipurpose tropical plant belonging to the family of *Moringaceae* (order: brassicales), with special reference to lectins and glycosidases, it was found that seeds of *Moringa oleifera* contain significant levels of α-mannosidase activity. This plant grows quickly even on soils having relatively low humidity. It is known to be non-toxic to humans and animals. It is reported to contain coagulant protein, which has much practical significance [Gassenschmidt *et al.,* 1995].

In the present work we describe the purification and characterization of α-mannosidase from *Moringa oleifera* present in the seed kernel. Furthermore, we also obtained information about the transition state analogues inhibition studies to this enzyme which will help in homology modeling.

4A.2.0 Materials and methods

4A.2.1. Materials

Moringa oleifera seeds (PKM_1) variety was purchased from the local market. *p*-Nitrophenyl-α-D-mannopyranoside, other *p*-nitrophenyl glycosides, α-methyl-D-mannopyranoside, Sephacryl S-200 HR and phenyl-Sepharose CL-6B was obtained from Fluka (Sigma-Aldrich), Con A Sepharose 4B gel supplied by Amersham (GE Healthcare), Uppsala, Sweden, DE-52 (DiEthyl cellulose) was obtained from Whattman, Ready to use standard protein molecular weight marker mixture for SDS-PAGE was obtained from Fermentas. Synthetic glyco-conjugates used in these studies were kindly provided by C. P. Rao, Department of Chemistry, IIT Bombay. All other chemicals and reagents were of analytical grade and procured from reputed firms.

4A.2.2. Enzyme assays

Under the standard test conditions, α-mannosidase activity was measured by the release of *p*-nitrophenol from the chromogenic substrate *p*-nitrophenyl-α-D-mannopyranoside (*p*NP-α-Man) 5 mM, stock. An assay mixture (500 µL) consisting of a 100 mM acetate buffer pH 5.0, 100 µL of a 5 mM *p*-nitrophenyl-α-D-mannopyranoside (1 mM final concentration) and the enzyme solution, was incubated at 37°C for 30 min. The control contained all reactants except the enzyme. Determination of other *p*-nitrophenylglycosidase activities was carried out under the same experimental conditions. The reaction was stopped after 30 min of incubation by addition of 1 M Na_2CO_3 buffer pH 10.0 to the reaction mixture. Liberated *p*-nitrophenol was measured spectrophotometerically at 405 nm.

The activity of the enzyme (units/ml/min) was calculated according to the formula given below:

$$\text{Activity (units/mL/min)} = \frac{\text{Absorbance at 405nm}}{\text{Time of incubation x 18.5 x Volume of enzyme}}$$

Where 18.5 is the molar extinction coefficient of *p*-nitrophenol

Definition of enzyme unit: One unit of enzyme activity was defined as amount of enzyme hydrolyzing 1 µmoL of substrate per min. The specific activity of the enzyme was expressed as units per mg protein. (IU/mg). All assays were performed in triplicate and results were recorded as the mean of these experiments.

4A.2.3. Extraction of *M.oleifera* α-mannosidase from seeds

Unless otherwise stated, all the purification steps were carried out at 4°C. Protein was monitored in the column fractions of various chromatographic steps by measuring the A_{280} in spectrophotometer. Enzyme activities were also monitored as described above.

Seeds of *M.oleifera* (PKM_1) were deshelled just before the extraction and the kernel was ground using a kitchen blender. Seed powder was defatted using chilled acetone, after removal of acetone by centrifugation the solids were air dried at room temperature. Total protein from 50 g of seed powder was extracted overnight with ten volumes of 25 mM Tris-HCl pH 7.4. After extraction the homogenate was centrifuged at 12,000 rpm for 30 min. The pellet was discarded and the supernatant which was considered as the crude extract of the enzyme preparation was subjected to 0-40% ammonium sulphate precipitation. After pelleting at 12,000 rpm for 30 min, the precipitated protein was collected and the supernatant was again saturated to 40-80% ammonium sulphate. The fraction of 40-80% precipitate which is found to be rich in α-mannosidase activity was dissolved in 25 mM Tris-HCl pH 7.4 and dialysed against same buffer.

4A.2.4. Anion exchange chromatography on DE-52

The 40-80% ammonium sulfate precipitated enzyme sample after dialysis was then loaded on to the DE-52 (4 x 9 cm), that has been previously equilibrated with 25 mM Tris-HCl pH 7.4. The unbound proteins were removed from the column by washing with five column volumes of the same equilibrating buffer. The absorbed proteins were then eluted using stepwise gradient of NaCl from 0.05–3 M NaCl in the same buffer. Fractions (1 mL each) were collected at a flow rate of 60 mL/h and assayed for the enzyme activity. The active fractions containing α-mannosidase were pooled and concentrated by Amicon concentrator.

4A.2.5. Hydrophobic interaction chromatography using phenyl-Sepharose CL-6B

The pooled and concentrated enzyme rich fractions from the previous step was saturated to a final concentration of 1.0 M with ammonium sulfate and applied on a phenyl-Sepharose CL-6B column (1.5 x 5 cm) previously equilibrated with 25 mM Tris-HCl pH 7.4 containing 1 M $(NH_4)_2SO_4$. The column was washed with equilibration buffer and the bound proteins were then eluted with 25 mM Tris-HCl pH 7.4. Fractions of 1 mL were collected at a flow rate of 1mL/min and active fractions were pooled together. The pooled fractions were concentrated and were dialysed in 25 mM Tris-HCl pH 7.4 buffer.

4A.2.6. Affinity purification on Con-A Sepharose 4B column

The concentrated fractions from phenyl-Sepharose were applied on to a Con A-Sepharose 4B column (5 ml) previously equilibrated with 25 mM Tris-HCl pH 7.4 containing 0.5 M NaCl (Equilibration buffer). After washing the unbound protein bound α-mannosidase was eluted with 0.4 M α-methyl-D-mannopyranoside in the same equilibration buffer at 10 mL/h. The elution of protein is monitored by checking absorbance at 280 nm as well as by checking the enzyme activity.

4A.2.7. Gel exclusion chromatography on sephacryl S-200 column

The α-mannosidase activity rich fractions from the Con A column were concentrated using centricon and applied onto a Sephacryl S-200 HR column (1 x 70 cm) previously equilibrated with 25 mM Tris-HCl pH 7.4, containing 150 mM NaCl. The protein was eluted at a flow rate of 10 mL/h. Fractions of 1ml was collected. The fractions that contain α-mannosidase activity were pooled and concentrated using Millipore Centriplus YM-30. This enzyme is used for all studies carried in this chapter.

4A.2.8. Protein estimation

Protein concentrations were determined by using commercially available Bradford dye reagent (from sigma), using BSA as the standard [Bradford, M. M., 1976].

4A.2.9. Estimation of carbohydrate content

The neutral sugar content of the purified enzyme preparation was determined by phenol sulfuric acid method of Dubois *et al.*, [1956] using glucose as standard.

4A.2.10. Native molecular weight determination

Native molecular weight of purified *M.oleifera* was determined using Sephacryl S-200 HR column (1 x 70 cm) size exclusion chromatography, according to the method described by Andrews [1964] Before loading the protein sample the column was calibrated with proteins of known molecular weight viz., Catalase (250 kDa), Alcohol Dehydrogenase (150 kDa), Phosphorylase (96 kDa), BSA (66 kDa). The protein was eluted at a flow rate of 10 mL/h. Fractions of 1ml was collected. The protein elution profile was monitored by absorbance by A_{280}. The graph was plotted as log MW versus V_e/V_o. Where: V_0 - Void volume, Ve - Protein elution volume.

4A.2.11. SDS-PAGE and sub unit molecular masses

To check the homogeneity and determine subunit molecular weight, the purified enzyme was analyzed using SDS-PAGE (stacking gel 5% and separating gel 10%) under reducing and nonreducing conditions [Laemmli U. K. 1970], using Fermentas unstained markers as standards. The gels were stained with Coomassie Brilliant Blue R-250. The subunit molecular mass of the purified α-mannosidase was determined according to Weber and Osborn [1969]. Relative mobility (R_f) of the denatured proteins on the gel was calculated as per formula given below. The molecular mass of the α-mannosidase was determined by interpolation from a linear semi logarithmic plot of log molecular mass of standard markers versus R_f values (Relative mobility).

$$\text{Mobility} = \frac{\text{Distance of protein migration x Length before staining}}{\text{Length after staining x Distance of dye migration}}$$

4A.2.12. Periodic acid Schiffs staining (PAS)

To determine the carbohydrate nature of the protein qualitatively, periodic acid - schiffs staining was carried out following the method of Zacharius *et al.*, [1969], with little modifications. Ready to use Schiffs reagent was procured from SRL. SDS-PAGE gel was stained in 1% periodic acid in 3% acetic acid for one hour. The gel

was washed for one hour with water and stained in Schiffs reagent for 30 minutes in dark. It was then destained with 10% acetic acid and finally stored in 3% acetic acid.

4A.2.13. Activity staining

Activity staining was carried out according to Blom *et al.*, [2008] to determine position of the band responsible for the α-mannosidase activity in native gels. The native PAGE was run with identical samples in different lanes. The bands corresponding to the silver staining in the native PAGE was cut into pieces and incubated in an eppendorf with substrate and performed enzyme activity under standard conditions. By comparing the part of the gel that showed the enzyme activity with the stained gel, the position of the enzyme was identified in the gel.

4A.2.14. Effect of pH and pH stability

The effect of pH on enzyme activity was determined at 37°C within a pH range of 2 to 8, using 0.1 M Citrate buffer (pH 2-3), 0.1 M NaOAc (pH 4-5), 0.1 M Sodium phosphate (pH 6-7), 0.1 M Tris-Hcl (pH 8). Stability of enzyme at various pH is determined by incubation the enzyme at various pH ranging from pH 2-8 at 37°C for 12 h. After incubation the residual enzyme activity was subsequently assayed under standard assay conditions.

4A.2.15. Effect of Temperature and Thermal Stability

Determination of optimum temperature for the *M.oleifera* α-mannosidase was performed with *p*-nitrophenyl-α-D-mannopyranoside (5 mM) in 100 mM NaOAc buffer pH 5.0 using incubation temperatures in the range of 30°C to 90°C. Thermal stability was determined by incubating the enzyme at 50°C, 60°C, 70°C, 80°C for 60 min, an aliquot was drawn at regular interval and immediately cooled. The residual enzyme activity was determined by standard assay method.

4A.2.16. Effects of EDTA and metal ions

The effect of EDTA and various divalent metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, CO^{2+}, Cu^{2+}, Hg^{2+}, Ag^{2+}, Fe^{2+}) on the activity of the α-mannosidase was carried according to Bloom *et al.*, [2008]. The effect of different metal ions on α-mannosidase activity was tested both with and without removal of preexisting ions by EDTA treatment. After EDTA treatment the enzyme is dialysed against NaOAc buffer pH 5.0. The effect of metal is tested by pre-incubating the enzyme in 100 mM NaOAc buffer pH 5.0

containing 1 mM concentration of each metal ion for 1 h prior to addition of the substrate (for EDTA 10 mM), and the residual enzyme activity was assayed by standard method. The enzyme activity without any metal ion is considered as a control (100%).

4A.2.17. Effect of other chemical agents

The effect of product analogues (mannose), simple sugars, reducing agents, β-mercaptoethanol, DTT, anionic detergent SDS, on enzyme activity was determined by pre-incubating the enzyme in sodium acetate buffer pH 5.0 containing at 37°C for 30 min. Later the remaining enzyme activity was assayed. The enzyme activity without any chemical agent is considered as a control (100%).

4A.2.18. Substrate specificity

The relative substrate specificity of α-mannosidase towards various synthetic substrates was determined. Substrates were prepared in 100 mM sodium acetate buffer pH 5.0 at final concentration of 5 mM (stock solution). The reaction was carried out using fixed concentration of enzyme and substrate under standard assay conditions. Relative activity on various substrates is expressed as percentage of the activity calculated with *p*-nitrophenyl α-D-mannopyranoside as a substrate (100%) with which enzyme showed maximum activity.

4A.2.19. Kinetic parameters determination

The Michaelis–Menton kinetic parameters (K_M and V_{max}) were determined by incubating the enzyme at optimum temperature/pH with different concentrations of substrate.

4A.2.20. Chemical modification of tryptophan residue using *N*-bromosuccinimide (NBS)

Chemical modification studies were carried out to get information about the tryptophan involvement in the active site of the enzyme. The tryptophan modifier, NBS 10 mM, (prepared in 100 mM sodium acetate buffer pH 5.0 was added in increments of 2 µL each time to the purified enzyme (0.3 mg/mL). After addition of this the residual enzyme activity was determined using the standard enzyme assay. The changes associated with the steady-state fluorescence of α-mannosidase due to NBS modification was monitored both in presence and absence of substrate (α-

methyl-D-mannopyranoside) using Perkin Elmer LS 55 fluorescence spectrophotometer, excitation at 280 nm and emission spectra was recorded in the range of 310-450 nm. The fluorescence spectra were measured at room temperature with a 1-cm path length cell. The monochromator slit width was kept at 1.5 nm in excitation and emission measurements.

4A.2.21. Effect of mannosidase specific inhibitors on the activity of α-mannosidase from *Moringa oleifera* seeds

To determine the class to which *M.oleifera* α-mannosidase is belongs two mannosidase specific inhibitors were used. Effect of two inhibitor were tested Deoxymannojirimycin (DMNJ) and Swainsonine (SW) a class I and class II inhibitors respectively, by incubating the enzyme with these inhibitors for 30 min and the residual enzyme activity was determined using standard assay.

4A.2.22. Inhibitory studies using transition state analogs

Preparation of glycoconjugates (inhibitors) and enzyme assay was carried according to Kumar *et.al*, [2009] using C1-/C2-aromatic-imino-glyco-conjugates of D-galactose, D-mannose and D-glucose (Glu2SI-Glucosyl Salicilyl Imine, GSI-Galactosyl Salicilyl Imine, GNI-Galactosyl Naphthyl Imine, Glu2NI-Glucosyl Naphthyl Imine, MNI-Mannosyl Naphthyl Imine) taking 50 µL of accordingly diluted purified enzyme with increasing amounts of the glycosidase inhibitor (i.e.; from 0 to 3 mM final conc.) at 37°C for 20 min. Enzyme assay was performed under standard assay condition. The activity without the inhibitor was considered as a 100% and the remaining activities at each concentration of inhibitor were determined w.r.t this value.

4A.2.23. Immunological studies

Ouchterlony double immunodiffusion was performed with 1% agarose gel in PBS as described by Ouchterlony [1948]. The agarose was poured into glass slide. Wells were punched in the agarose. In one well *M.oleifera* α-mannosidase was loaded and in the other well jack bean α-mannosidase antibody (available in our lab). A precipitin band was allowed to develop at 37°C for 24-48 h in humidified chamber.

4A.3.0. Results

The Glycosidase activities in the crude Tris-HCl buffer pH. 7.4 extract of *M.oleifera* seeds were examined using various *p*NP-glycosides as substrates. As shown in the Table: 4A.1, among various glycosidase it mainly contains α-mannosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and β-hexosaminidase.

4A.3.1. Purification of α-mannosidase

The purification of α-mannosidases from *M.oleifera* seeds is summarized in Table: 4A.2. Nearly 36.6 fold purification with 6.2% yield was obtained through this purification process. The crude extract was concentrated using ammonium sulphate 0-40% and 40-80%. *M.oleifera* α-mannosidase activity was observed in the second step of ammonium sulphate precipitation (40-80%). After dialysis and loaded on to DE-52 column (anion exchanger) it was observed that α-mannosidases from *M.oleifera* is completely retained on DE-52 column in Tris-HCl buffer at pH 7.4. After washing the unbound proteins the α-mannosidases was specifically eluted in stepwise manner by including NaCl in the Tris-HCl buffer pH 7.4. In DE-52 column the highest α-mannosidase activity was seen in the 100 mM NaCl elution fraction [Figure: 4A.1-A]. This fraction is used for further purification of the α-mannosidases. The enzyme rich fractions when concentrated and passed through phenyl Sepharose column (hydrophobic chromatography), enzyme is retained at high concentration of ammonium sulphate and eluted in absence of this salt [Figure: 4A.1-B]. Partially purified *M.oleifera* α-mannosidase was found to be a glycoprotein confirmed from its ability to bind to Con A-Sepharose 4B gel, When α-mannosidase rich fractions of the phenyl- Sepharose chromatography was loaded on to the Con A Sepharose *M.oleifera* α-mannosidase was strongly bound, which is later eluted specifically by using 0.4 M methyl-α-mannopyranoside in 25 mM Tris-HCl pH 7.4 containing 0.5 M NaCl [Figure: 4A.2-A]. Con A eluted enzyme rich fractions are pooled, concentrated and was further purified to homogeneity using S-200 gel filtrations, which give a one major symmetrical peak of α-mannosidase activity coinciding with protein peak [Figure: 4A.2-B]. The same column is also used for determining the native molecular mass of the intact enzyme after calibrating the column with standard proteins of known molecular weights.

4A.3.2. Molecular properties of the *M.oleifera* α-mannosidase

The purified native *M.oleifera* α-mannosidase exhibited a molecular mass of ~230-240 kDa on S-200 gel filtration [inset Figure: 4A.2-B]. In 10% SDS-PAGE, the enzyme dissociated into two subunits with molecular masses of 66 kDa (α-larger subunit) and 55 kDa (β-smaller subunit), under both reducing and non-reducing conditions, calculated with relative migration compared to the protein markers [Figure: 4A.3-A & B]. An additional band at 116 kDa was also observed which has been discussed in the later section. Under the PAS staining of the SDS-PAGE run gel it was observed that only the larger subunit got stained with the Schiffs reagent indicating, larger subunit got glycosylated. The activity assay for the native PAGE confirms the band responsible for the α-mannosidase activity [Figure: 4A.4-A, B, C & D]. The purified α-mannosidase was found to be a glycosylated protein with 9.3% carbohydrates as estimated by phenol sulphuric method.

4A.3.3. Biochemical properties of *M.oleifera* α-mannosidase

The purified *M.oleifera* α-mannosidase showed optimal enzyme activity at pH 5.0 [Figure: 4A.5-A]. Purified α-mannosidase from *M.oleifera* showed stability from pH 3 to 7 with more than 80% of the enzyme activity remained at this pH, after 12 h of incubation [Figure: 4A.5-B]. The temperature optimum of this enzyme is at 50°C. [Figure: 4A.5-C]. More than 80% of activity is seen from 40 to 60°C. Thermostability of enzyme was examined by incubation the enzyme at various temperatures and the residual enzymatic activity was measured at regular intervals for 60 min. As it can be observed from the [Figure: 4A.5-D], the result indicate more than 70% of the activity is retained after incubation at 50°C for 60 min. Enzyme completely lost its activity at 80°C after 1h of incubation and only 26%, and 12% activity is remained at 60 and 70°C, respectively.

4A.3.4. Effect of various metal ions, sugars and chemical reagents

The effect of different divalent cations and EDTA on α-mannosidase activity was tested both with and without removal of pre-existing ions by EDTA treatment. The results are summarized in Table: 4A.3. EDTA treatment leads to 56 % loss of the activity after 1 h of incubation in the acidic medium. Prolonged incubation leads to complete loss of the activity. This activity is completely restored by Zn^{2+} only, in EDTA treated samples. In presence of Cu^{2+}, Hg^{2+} and Ag^{2+} 97%, 90% and 92% of the

enzyme activity was lost respectively, in non-EDTA treated samples. However, other metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} did not show any significant loss of activity. Effect of simple sugars (mannose, glucose and galactose), reducing agents (β-mercaptoethanol and DTT) and detergent (SDS) on the activity of the α-mannosidase was examined using purified enzyme [Table: 4A.4]. The influence of the anionic detergent like SDS showed 100% inhibition on the enzyme activity at 1 mM concentration. 73% of the original activity was remained at 10 mM concentration of mannose, being a product analog it showed significant inhibition at higher then 50 mM concentration. Glucose and galactose showed 86% and 92% of the enzyme activity at 10 mM concentration. In presence of reducing agent like β-mercaptoethanol (1% v/v) light increase in the activity of about 10% was observed. DTT showed considerable decrease up to 27% in the enzyme activity at 1 mM concentration.

4A.3.5. Substrate specificity

The specificity of purified α-mannosidase was assayed in presence of synthetic substrates. No significant activities towards other para-nitrophenyl- glycosides were observed except *para*-nitrophenyl-α-D-mannopyranoside [Table: 4A.5]. The K_m and V_{max} value was measured using *p*-nitrophenyl-α-D-mannopyranoside and was found to be 1.6 mM and 2.2 U/mg respectively.

4A.3.6. Chemical modification studies

The α-mannosidase activity was completely inhibited at 1 mM of NBS. Modification of tryptophan residue by NBS resulted in total quenching of fluorescence. Modification of tryptophan residue by NBS also associated with the blue shift quenching of the fluorescence spectrum [Figure: 4A.6-A]. However, in presence of substrate (α-methyl-D-mannopyranoside) total quenching was protected [Figure: 4A.6-B].

4A.3.7. Effect of mannosidase specific inhibitors

The effect of mannosidase specific inhibitors such as deoxymannojirimycin (DMNJ) and swainsonine (SW) on enzyme activity is shown in Table: 4A.6. The enzyme activity was completely inhibited by SW at 0.001 mM (1 µM) concentration. Whereas, DMNJ at the same concentration did not influence the enzyme activity. At 0.5 mM concentration of DMNJ showed only 31% inhibition.

4A.3.8. Inhibition by glycoconjugates

The inhibition results of the glycoconjugates are shown in [Figure: 4A.7-A]. Among the tested different glycoconjugates naphthylidene-conjugates of mannose, glucose and galactose exhibit 100% inhibition at 1.0 mM, 1.5 mM, and 2.0 mM concentration respectively. The salicylidene-conjugates of the same sugars exhibit 100% at 3.0 mM concentration. Concentration at which a 50% inhibition of enzyme is brought (IC_{50}) by glyco-conjugates is shown in Figure: 4A.7-B.

4A.3.9. Immunochemical study

In order to investigate immunological relationships of the *M.oleifera* α-mannosidase with that of the jack bean α-mannosidase, Ouchterlony double immunodiffusion analysis was carried using antisera raised against jack bean α-mannosidase. An arc was observed between the two wells indicating that *M.oleifera* α-mannosidase cross-reacted with jack bean α-mannosidase antisera [Figure: 4A.8].

FIGURES

Figure: 4A.1-A & B.

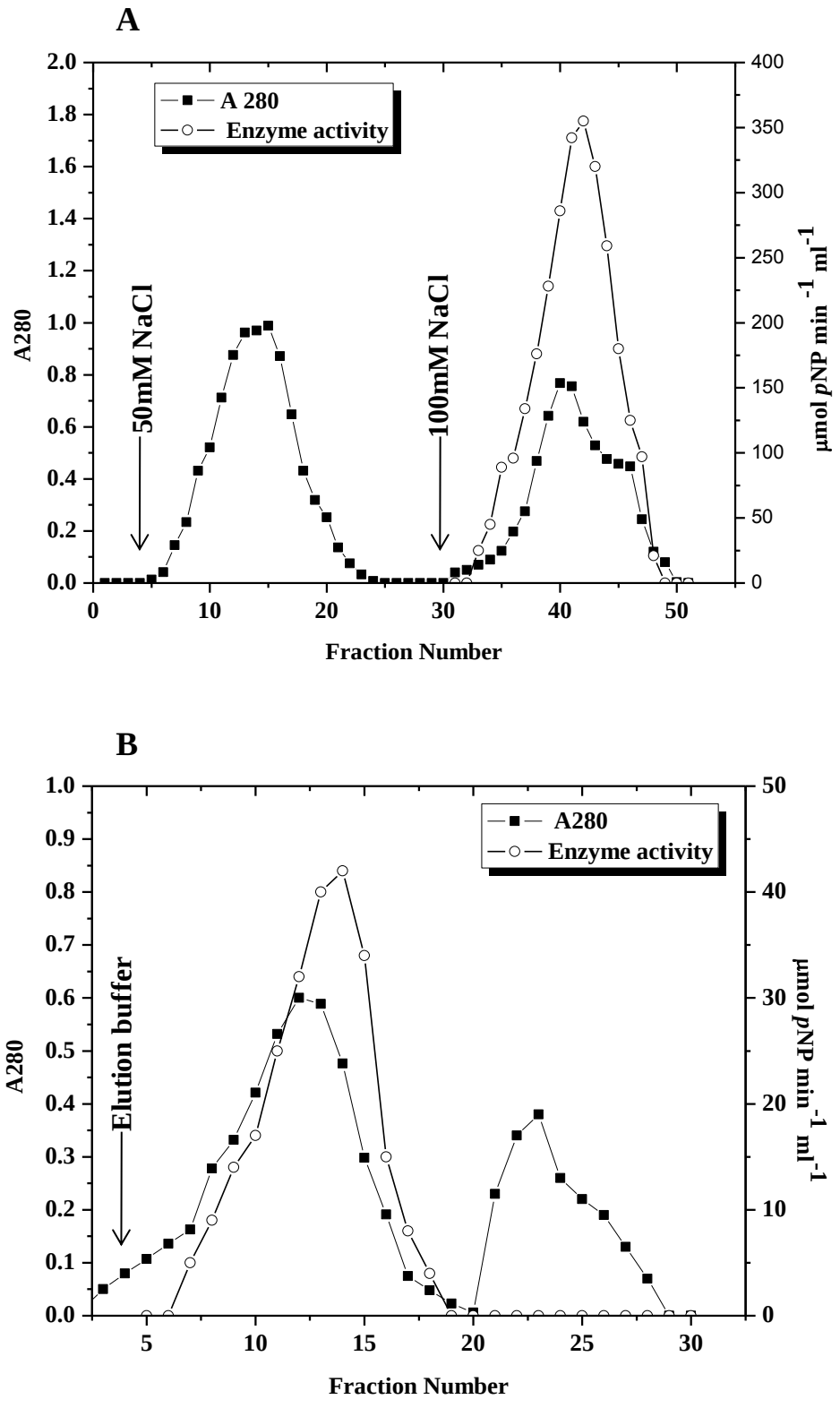


Figure: 4A.1 A & B. Elution profile of *M.oleifera* α-mannosidase on (A) DE-52 column and (B) phenyl Sepharose column: (A) The crude enzyme fraction of *M.oleifera* seeds after $(NH_4)_2SO_4$ precipitation and dialysis was applied onto the DE-52 column (4 cm X 9 cm). The column was washed with 25 mM Tris-HCl buffer pH 7.4 (equilibration buffer), and the bound enzyme was eluted by 100 mM NaCl in the same buffer. Fractions of 1 mL were collected and the absorbance monitored at 280 nm. Arrow indicates point of application of salt. **(B)** The enzyme rich fractions from the DE-52 were concentrated and saturated to 1 M $(NH_4)_2SO_4$ in equilibration buffer. After washing, the bound protein was eluted with buffer in the absence of $(NH_4)_2SO_4$.

Figure: 4A.2 A & B

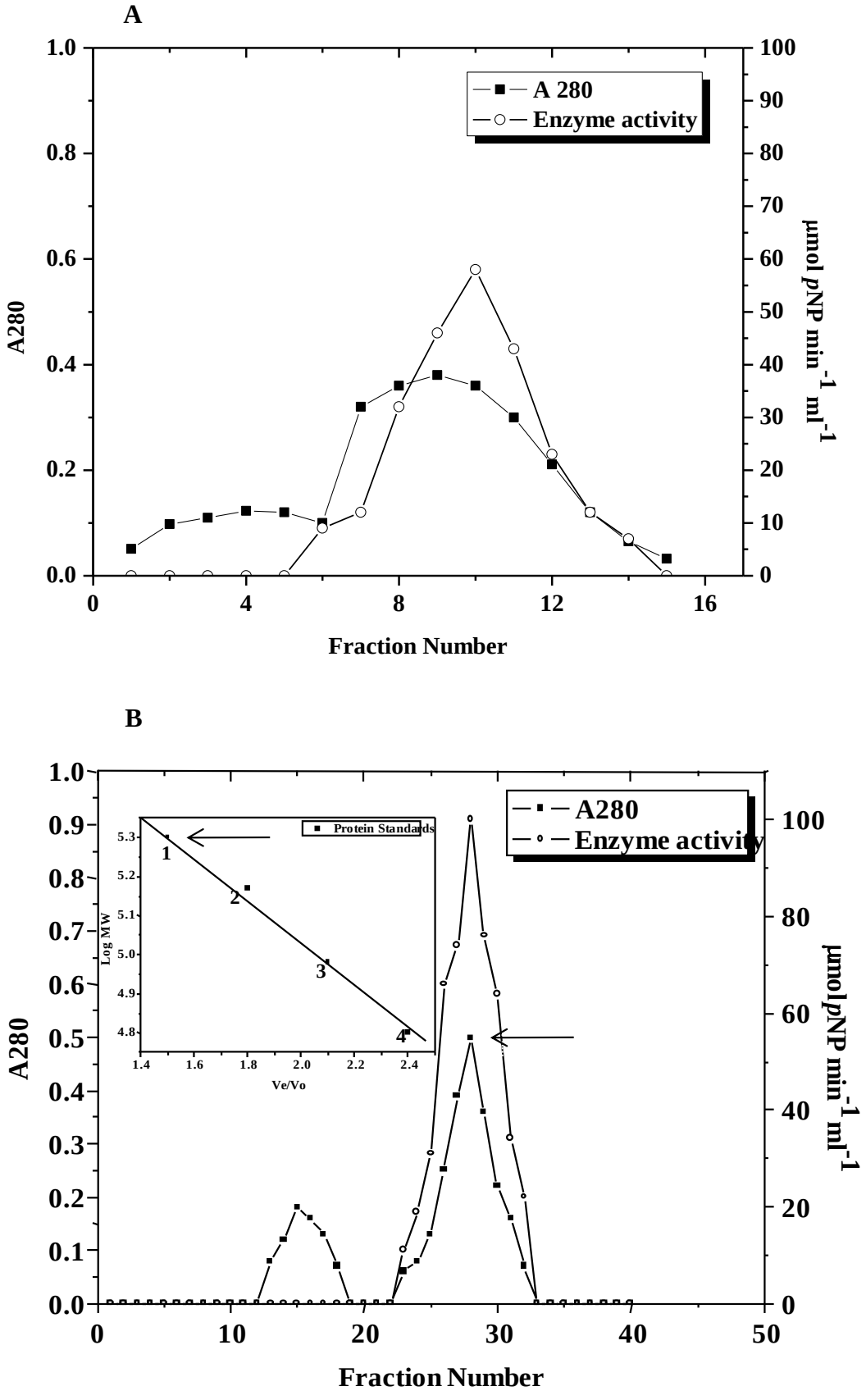
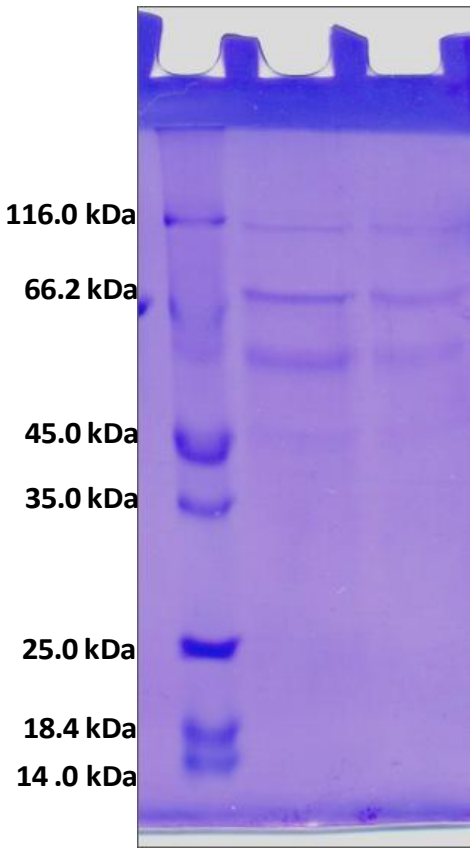
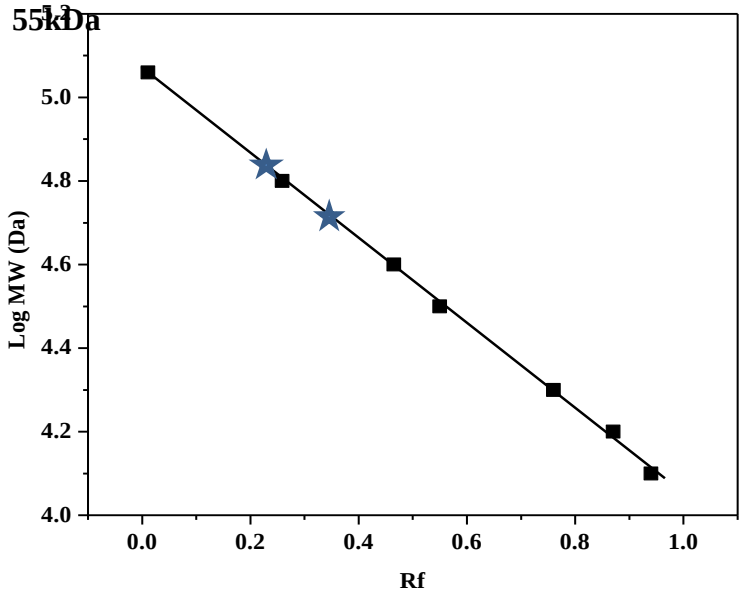


Figure: 4A.2A & B. (A) Elution profile of α-mannosidase from Con A column and (B) Gel filtration of the enzyme on S-200 gel and molecular weight determination: (A) DE-52 enzyme rich fractions were pooled and loaded on to the Con A-Sepharose 4B column (5 mL) previously equilibrated with 25 mM Tris-HCl pH 7.4 containing 0.5 M NaCl (Equilibration buffer). After washing the gel with equilibration buffer, the bound α-mannosidase was eluted with 0.4 M α-methyl-D-mannopyranoside in the same buffer. **(B)** The α-mannosidase eluted from Con-A gel was concentrated and loaded onto a Sephacryl S-200 column (1 x 70 cm). The protein was eluted at 10 mL/h with 25 mM Tris-HCl buffer pH 7.4 containing 150 mM NaCl. The eluted fractions were checked for the enzyme activity. The same column was also calibrated using standard proteins of known molecular weights 1.Catalase (250 kDa), 2. Alcohol Dehydrogenase (150 kDa), 3. Phosphorylase (96 kDa) and 4. BSA (66 kDa). Ve/Vo values were plotted against Log molecular weight of the protein. Arrow indicates the point where *M.oleifera* α-mannosidase was eluted.

Figure: 4A.3A & B.



A



B

Figure: 4A.3 A&B.

A) **10% SDS-PAGE of purified *Moringa oleifera* α-mannosidase:** Lane M: Standard molecular weight markers, Lane 1 & 2: Purified α-mannosidase under both reducing and non reducing conditions respectively. Gels were stained with Coomassie Brilliant Blue. The standard protein molecular weight markers used were; β- galactosidase (166.0 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), lactate degyhydrogenase (35.0 kDa), REase Bsp 981 (25 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa) (Fermentas).

B) **Determination of Subunit molecular weights of *Moringa oleifera* α-mannosidase:** The mobility of the protein was determined as per the formula given in the text. The values are plotted against the log MW (Da) Vs R_f values.

Figure: 4A.4A, B, C & D

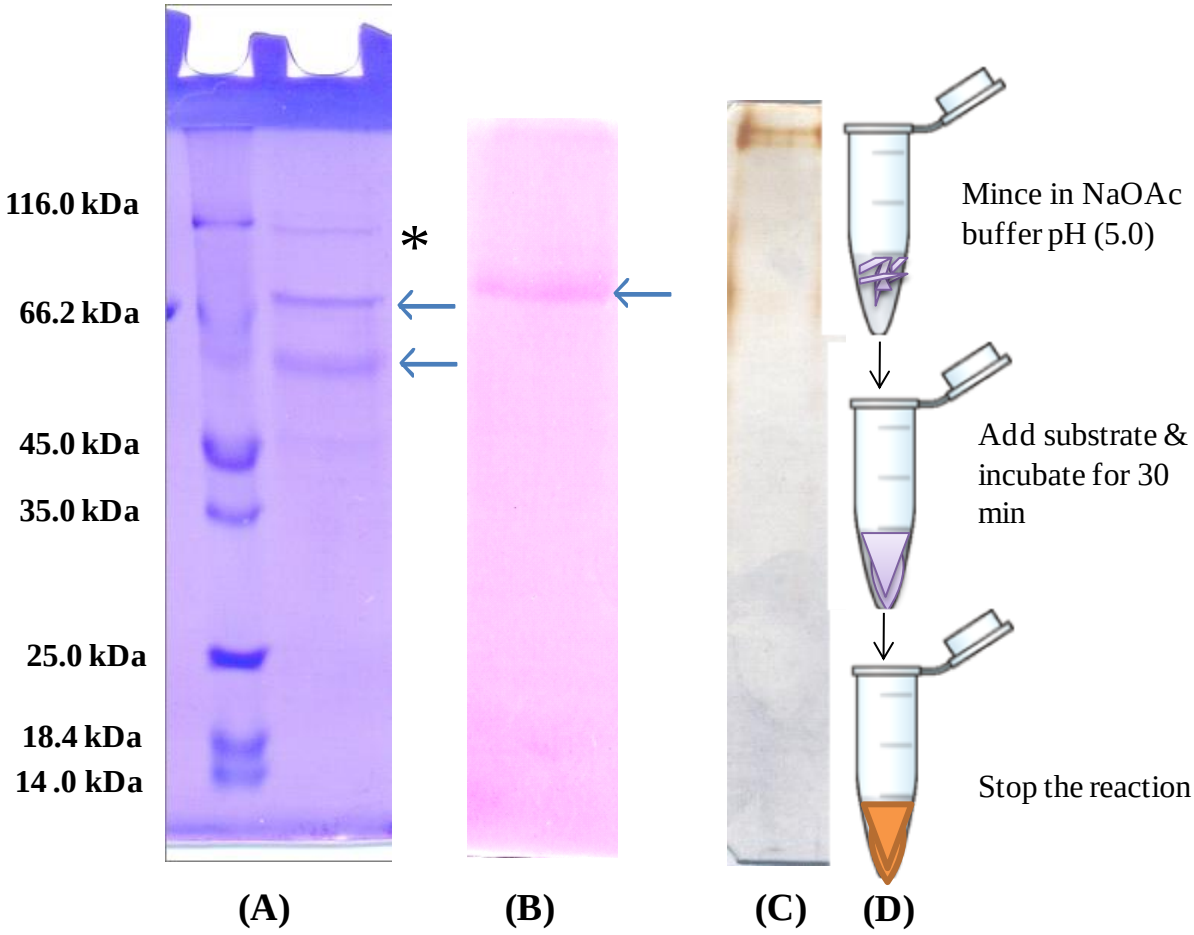


Figure: 4A.4A, B, C & D. (A) 10 % SDS-PAGE , (B) Periodic acid schiffs (PAS) staining, (C) Native PAGE activity staining and (D) Activity assay of *M.oleifera* α-mannosidase: (A) 10% SDS-PAGE of purified *M.oleifera* α-mannosidase (showed for comparison). **(B)** The same gel was run in duplicates under reducing conditions and stained with Schiffs reagent (PAS staining). Arrow corresponds to the larger subunit (66 kDa) protein in the SDS-PAGE. **(C)** Native-PAGE of the purified *M.oleifera* α-mannosidase used for the activity staining. **(D)** The corresponding band of the native - PAGE run in duplicate was used for activity assay after incubation with both buffer and substrate for 30 min reaction was stopped and the color change observed.

*Indicates possible 116 kDa precursor form of mannosidase containing mannose binding domains. (As identified with MALDI-TOF; MS/MS analysis)

Figure: 4A.5. A, B, C & D.

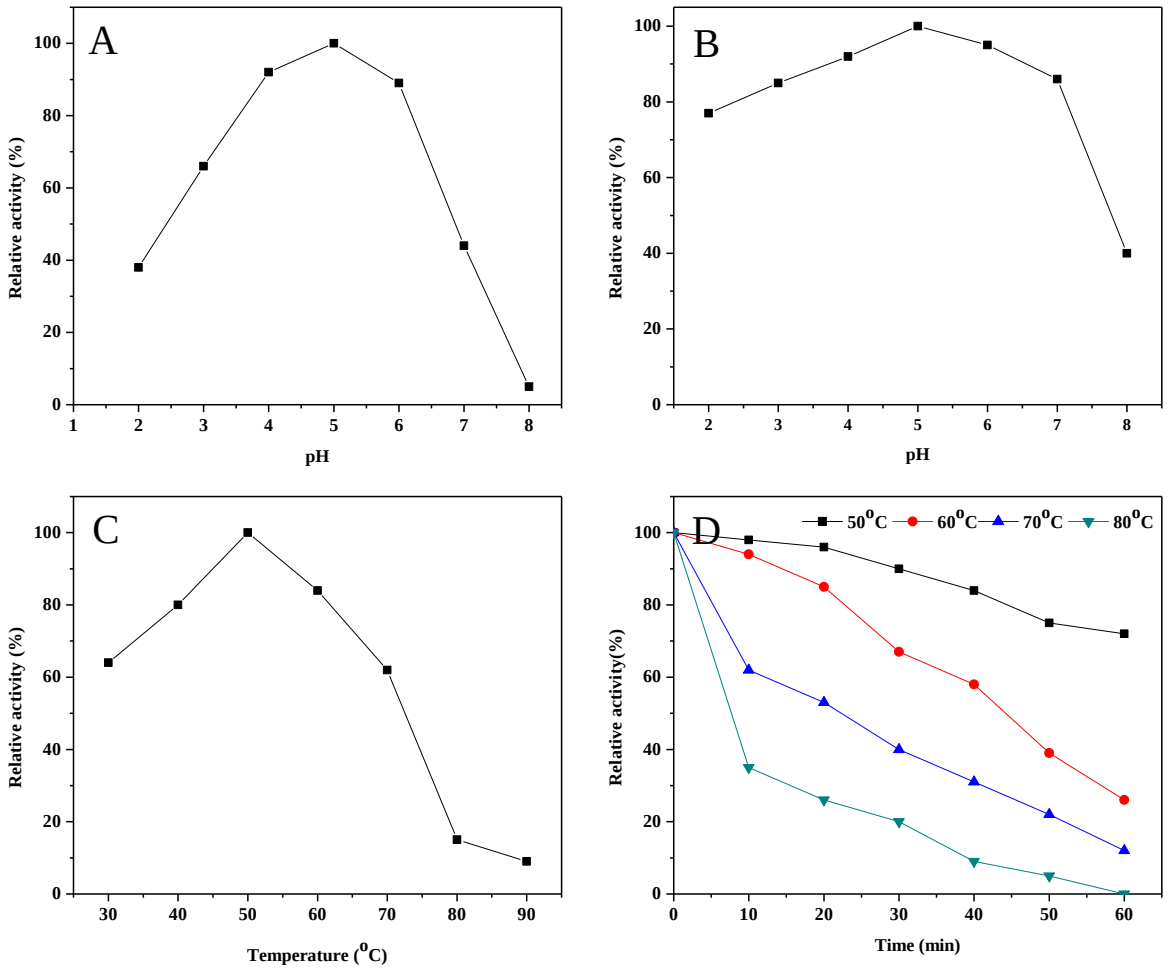


Figure: 4A. 5. A, B, C & D. Characterization of the enzymatic properties of purified α-mannosidase from *Moringa oleifera*. (A) Effect of pH on the α-mannosidase activity of *M.oleifera* was determined at 37°C in buffers ranging from pH 2.0 to 8.0. The value obtained at pH 5.0 where α-mannosidase activity is maximum was taken as 100%. **(B)** pH stability of *M.oleifera* α-mannosidase was determined by measuring α-mannosidase activity under standard assay conditions (*p*NPM) after pre-incubation of the enzyme at 37°C for 12 h in buffers ranging from pH 2.0 to 8.0. The activity of an untreated enzyme sample at pH 5.0 was taken as 100%. **(C)** Effect of temperature on α-mannosidase activity was determined in 100 mM NaOAc buffer (pH 5.0) at 30–90°C. The value obtained at 50°C was taken as 100%. **(D)** Thermostability of α-mannosidase was determined by measuring α-mannosidase activity under standard assay conditions after pre-incubation of the enzyme in 100 mM NaOAc buffer (pH 5.0) at 50, 60, 70 and 80°C for various periods. The activity of an unheated enzyme sample was taken as 100%. Data is the mean of triplicate experiments.

Figure: 4A.6 A & B

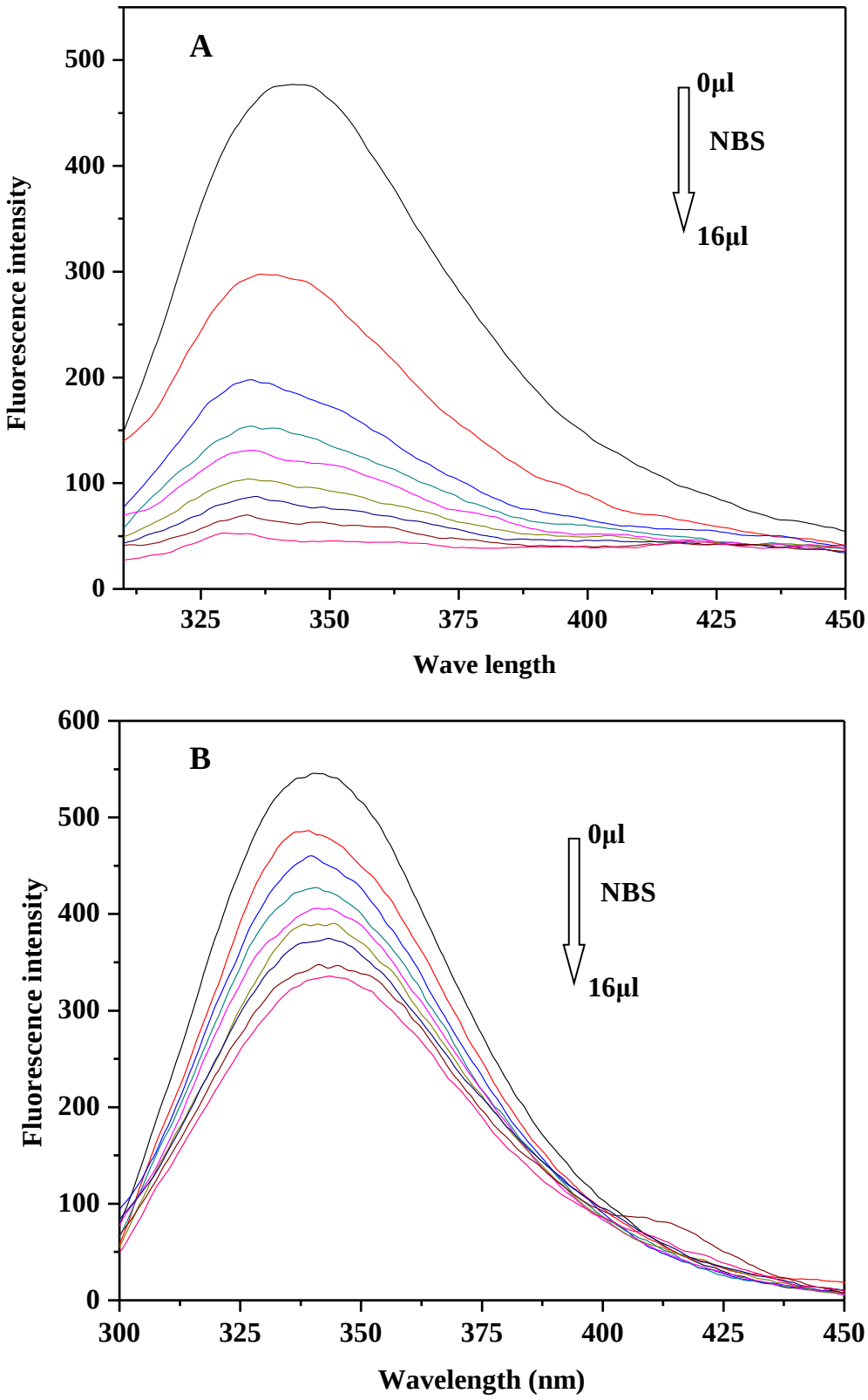
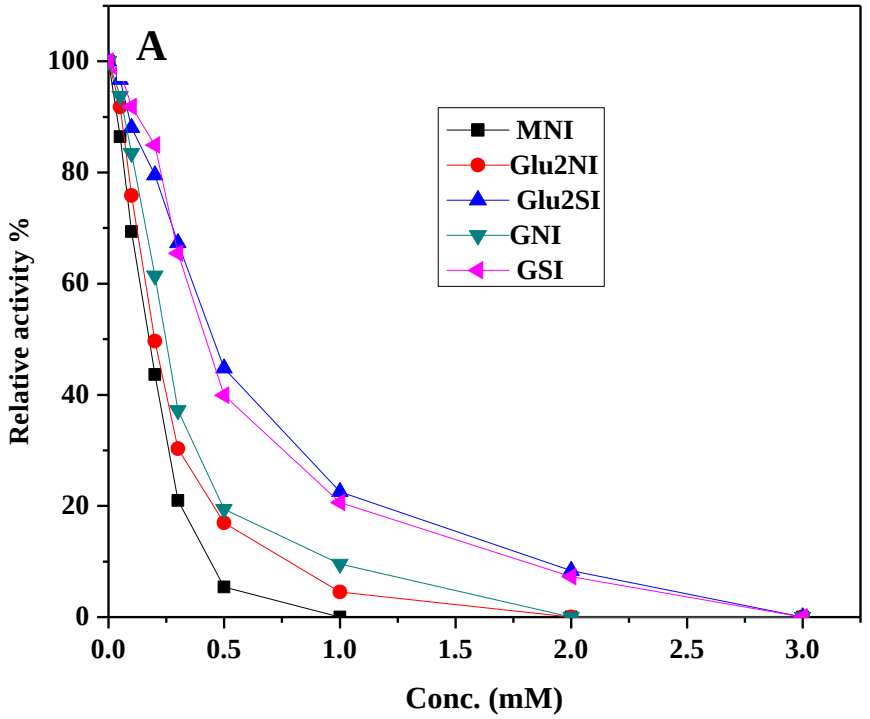


Figure: 4A.6 A & B. Influence of NBS on *Moringa oleifera* α-mannosidase activity: (A) Fluorescence spectra of *M.oleifera* in absence and **(B)** in presence of substrate. 10 mM NBS (prepared in 100 mM sodium acetate buffer pH 5.0 was added each time in the increment of 2 μL each time. The Fluorescence was recorded by excitation at 280 where, maximum emission was observed and emission was recorded in the range of 310-450 nm.

Figure: 4A.7 A & B



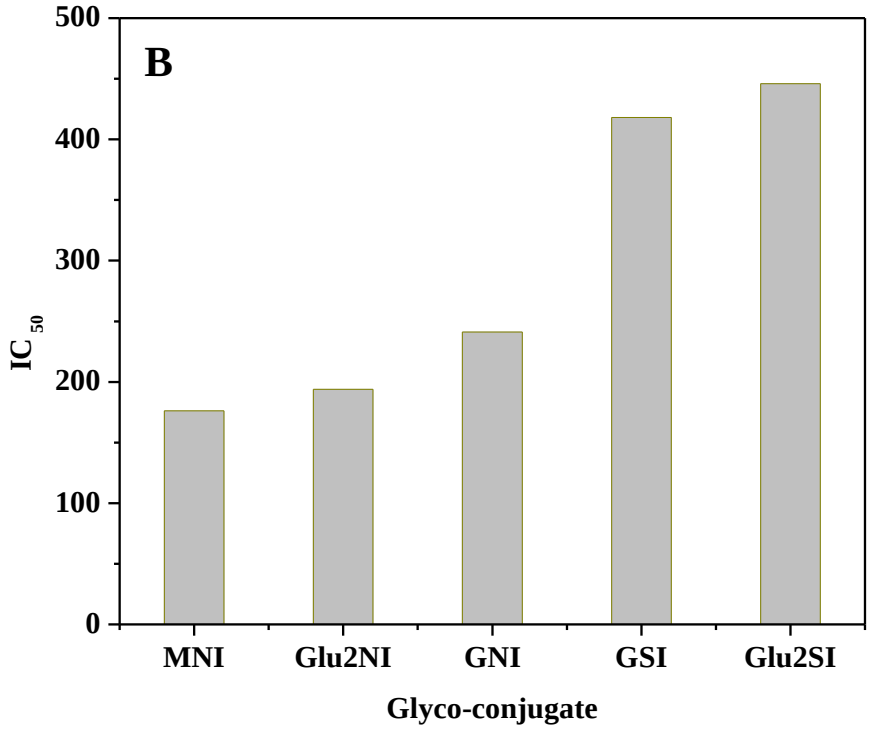


Figure: 4A.7 A & B. (A) Inhibition of enzyme activity with different Glyco-conjugates, (B) IC_{50} values: (A) C1-/C2-aromatic-imino-glyco-conjugates of D-galactose, D-mannose and D-glucose: the purified α-mannosidase from *M.oleifera* was incubated with these glyco-conjugates for 20 min. The structures of the synthetic glyco-conjugates used in this study are given in figure.3.4. Enzyme assay was performed using standard assay conditions. The activity without the inhibitor was considered as a 100%. The relative activity at each concentration was measured. **(B)** IC_{50} values of these glyco-conjugates. Each value is mean of three independent readings.

Figure: 4A.8

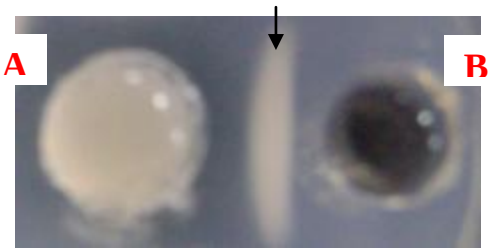


Figure: 4A.8. Immunological cross-reactivity of *Moringa oleifera* α-mannosidase with the Jack bean α-mannosidase antiserum: Well A: Jack bean α-mannosidase antibody and Well B: *Moringa oleifera* α-mannosidase. Arrow indicates the precipitin line (Arc).

TABLES

Table: 4A.1. Various glycosidase activities in the crude *M.oleifera* seeds extracted in 25 mM Tris-

Glycosidases	Enzyme activity (U/mg)[a]
α-mannosidase	28.7
α-galactosidase	29
β-galactosidase	14
α-glucosidase	20
β-glucosidase	16
β-hexosaminidase	13.7

[a] The enzyme units were calculated for 1g of the seed tissue. The enzyme activities were measured at pH 5.0 using *p*NP-glycosides (5 mM) as substrates as described under methods.

Table: 4A.2. Purification of α-mannosidase from *Moringa oleifera* Seeds

Purification step	Total Protein (mg)	Total activity [b] (units)	Specific activity [c]	Yield (%)	Fold purification
Crude extract [a]	1440	1435	0.99	100	1
40-80% Ammonium sulfate	520	1224	2.3	85.2	2.3
DE-52 cellulose	120	846	7	58.5	7.07
phenyl-Sepharose CL-6B	15	425	28.3	29.6	28.5
Con-A Sepharose	7	220	31.4	15.3	31.7
Sephacryl- S 200	2.5	90	36	6.2	36.6

[a] 50 g of defatted *Moringa oleifera* seed kernel powder was used at a time and the purification in different chromatographic steps was carried out in batch wise. Proteins were measured in chromatographic fractions using A_{280}.

b One unit of enzyme activity was defined as amount of enzyme hydrolyzing 1 µmol of *p*-nitrophenol from PNPG per min at pH 5.0 and 37°C.

c The specific activity of the enzyme is expressed as units per mg protein. (U/mg of protein).

Table: 4A.3. Effect of divalent metal ions and EDTA on the *Moringa oleifera* α-mannosidase activity.

Effector Agent	**Relative Activity (%)**	
	Non-EDTA treated	EDTA treated
Control	100	100
EDTA [a]	44	-----
Ca^{2+}	97	96
Mg^{2+}	92	81
Mn^{2+}	86	83
Zn^{2+}	101	203
Fe^{2+}	65	78
Cu^{2+}	3	0
Co^{2+}	36	45
Hg^{2+}	10	0
Ag^{2+}	8	0

[a] EDTA concentration 10 mM, all other metal ions 1 mM, untreated enzyme with metal ion is taken as control 100%.

Table: 4A.4. Effect of simple sugars, denaturing and reducing agents on *M.oleifera* α-mannosidase activity.

Chemical agent	Concentrations	Relative activity (%)[a]
Simple sugars		
Mannose	5mM	85
	10mM	73
	50mM	30
Glucose	5mM	94
	10mM	86
	50mM	46
Galactose	5mM	97
	10mM	92
	50mM	83
SDS (Anionic detergent)	1mM	ND
Reducing agents		
β-mercaptoethanol	1% (v/v)	110
DTT	1mM	73

[a] Relative activities are measured taking enzyme activity of *M.oleifera* α-mannosidase without adding any chemical agent as 100%. All the experiments are performed in triplicates and the mean values are represented. ND: Not Detected

Table: 4A.5. Relative Activity of *M.oleifera* α-mannosidase with various synthetic substrates.

Substrate [a]	Relative activity (%) [b]
***p*NP-α-D-mannopyranoside**	100
***p*NP-α-D-glucopyranoside**	0.22
***p*NP-β-D-glucopyranoside**	0.18
***p*NP-β-D-galactopyranoside**	0.12
***p*NP-α-D-galactopyranoside**	0.09

[a] All the synthetic substrate concentration used were 1 mM in the final reaction mixture.
[b] Relative activities were calculate in relation to *pNP*-α-D-mannopyranoside activity that was considered as 100%.

Table: 4A.6. Effect of mannosidase specific inhibitors on the activity of α-mannosidases from *Moringa oleifera* seeds.

Inhibitors	Concentration(mM)	Relative activity (%)
Control	0	100%
Swainsonine (Class II)	0.001	5%
	0.01	ND
	0.05	
	0.1	
	0.5	
Deoxymannojirimycin (Class I)	0.001	100
	0.01	99
	0.05	97
	0.1	78
	0.5	69

Results were the percentage relative activity compared to the control which is taken as 100%. ND: Not Detected.

4A.4.0. Discussion

In the frame work of our interest for new glycosyl hydrolases from the seeds of non-legumes we have successfully purified a class II α-mannosidase from *M.oleifera* seed kernels using conventional chromatographic techniques. To our knowledge there have been no reports on the purification of any glycosidase from *M.oleifera* seed kernels. The purification protocol involved four chromatographic steps anion exchange chromatography, hydrophobic interaction chromatography, Con-A lectin affinity chromatography, and S-200 gel permeation. In the first step of ammonium sulphate precipitation (0-40%) no detectable α-mannosidase activity was observed. This fraction contained largely small cationic proteins, which are abundant in *M.oleifera* [Gassenschmidt *et al.,* 1995]. The α-mannosidase activity was mainly observed in the 40-80% fraction. On DE-52 this enzyme is completely retained and was eluted with increasing concentration of the NaCl in the buffer. The step wise elution of the enzyme from the DE-52 also helps in separating the α-mannosidase from other contaminating proteins such as the α-galactosidase which, appears in the crude extract. The fact that the enzyme binds strongly to Con-A gel suggests its glycoprotein nature which is further confirmed by its specific elution from the gel using the sugar methyl-α-mannopyranoside. The single protein peak which overlaps with the enzyme activity peak suggests that it is a homogeneous preparation. The native molecular mass of the purified enzyme was approximately 230 kDa as determined by gel filtration. Native PAGE analysis of the enzyme revealed that it is homogeneous and the band exhibited enzyme activity. On the other hand, by SDS-PAGE under reducing and non reducing conditions the enzyme dissociated into two subunits of molecular mass 66 kDa and 55 kDa, suggesting that this enzyme to be tetrameric. It is comparable to Jack bean and tomato α-mannosidase. However, the subunit masses differ from these species. Jack bean enzyme has two subunits of 66 kDa and 44 kDa, whereas, tomato enzyme has subunits of 70 kDa and 47 kDa respectively [Snaith, 1975, Hossain, *et al.,* 2009]. Under both reducing and non reducing conditions the enzyme showed similar band pattern suggesting the interaction between the subunits to be non-covalent which is common feature as reported earlier [Tulasi and Nadimpalli, 1997; Kimura *et al.,* 1999; Mahadevi, *et al,* 2002; Hossain, *et al.,* 2009]. Monomeric forms of mannosidase were also observed in some plant species like *Artocarpus communis* seeds (Isoform I-75 kDa; II-61 kDa),

Erythrina indica seeds (127 kDa) [Kestwal and Bhide, 2005; Ahi *et al.,* 2007]. However *Ginko biloba* seeds α-mannosidase is 340 kDa in the native form and subunit of 120 kDa [Woo *et al.,* 2004]. In closely related members of *M.oleifera* order like tropical fruit babaco (brassicales) oligomeric form of α-mannosidase was observed [Blom *et al.,* 2008]. *Prunus serotina* Ehrh and *Medicago sativa* [Curdel and Petek, 1980; Waln and Poulton, 1987] also has the four-subunits with different molecular masses. *M.oleifera* is a glycoprotein as detected by periodic acid-Schiff staining. This was further confirmed to contain 9.3 % carbohydrate. This carbohydrate content is more than that of mannosidase from *Erythrina indica* seeds [Kestwal, *et al.,* 2007]. *M.oleifera* α-mannosidase when stained with PAS staining, it was observed that only the larger subunit got stained with the Schiffs reagent [Figure: 4A.4-B] indicating, larger subunit is glycosylated, it is also observed with jack bean α-mannosidase [Kimura *et al.,* 1999]. The purity of the α-mannosidase was verified by native PAGE (silver staining) and showed one homogeneous band [Figure: 4A.4-C]. The enzymatic identity of this band was verified by cutting identical unstained lanes of the same gel, equivalent to band in Figure: 4A.4-C, into different pieces and performing activity assays. By relating the presence of activity with the respective position on the gel, the band responsible for the α-mannosidase activity could be identified Figure: 4A.4-D. SDS-PAGE showed one additional band at 116 kDa region, which was consistently seen in all the purification steps. This protein band when subjected to MALDI analysis for the identification of the protein, it was confirmed to be 116 kDa glycoprotein containing a mannose binding domains in it [results not shown]. This results guide to interesting findings which are reported on class II α-mannosidase [Howard *et al.,* 1998]. In humans lysosomal α-mannosidase the initial enzyme is synthesized as a polypeptide of 110 kDa that is subsequently processed into two subunits of 40–46 kDa and 63–67 kDa, which then constitute the native protein (molecular mass, 210 kDa). The rat Golgi α-mannosidase II is a dimer composed of 124 kDa subunits. Treatment with chymotrypsin causes limited proteolysis to give a dimer of 110 kDa subunits that retains full activity. Some studies reported the possibility that the jack bean α-mannosidase is also synthesized as a polypeptide chain of 110 kDa, which forms a dimer. The fragments of mass 44 kDa and 66 kDa, observed by SDS-PAGE, could be due to limited proteolysis of the protein, which is still able to maintain its integrity unless exposed to denaturing conditions [Howard *et al.,* 1998].

The optimum pH of *M.oleifera* α-mannosidase enzymes was around pH 5.0 when tested with *p*NP-α-D-mannopyranoside as a substrate, similar to those of other plant α-mannosidases reported [Ahi *et al.,* 2007; Hossain, *et al.,* 2009]. Some enzymes have pH optimum between the range of pH 4.0-5.0 [Prijcker, *et al.,* 1974; Paus, 1977; Niyogi and Singh, 1988; Kishimoto *et al.,* 2001]. The optimal pH for the almond α-mannosidase was around pH 3.8 [Misaki *et al.,* 2003]. The enzyme was active at acidic pH and activity decreased as the pH approached the alkaline range pH 8.0. This is within the range of reported values of α-mannosidase from *Capsicum* and tropical fruit *Babaco* [Priya Sethu and Prabha, 1997; Blom *et al.,* 2008]. It is interesting to note that the activity of the glycosidases is high at acidic pH and the pH that naturally exists in the protein bodies of the seeds where these enzymes are co-localized with storage proteins, lectins. This suggests possible *in vivo* physiological significance of their co-localizations that may be important during seed growth and development.

The optimum temperature of this α-mannosidase was found to be 50°C, above this temperature, enzyme activity declined rapidly as the temperature increased, but the enzymes were not completely inactivated even at 80°C after 30 min of incubation. Further the thermal stability was studied by incubating the enzyme at different temperatures for 60 min. The enzyme is quiet stable to 50°C for 60 min with more than 75% of the activity retained. Even when it was incubated at 70°C for 40 min 30% of the original activity remained. This high temperature stability may be due to two reasons one is due to the tetrameric form which are stable compared to the monomeric and dimeric forms; the second reason is due to the high glycosylation of this enzyme.

Among several metal ions incubated with the EDTA treated sample, only Zn (II) is reported to restore the total activity of the enzyme. The study of inactivation of α-mannosidase by EDTA and its reversion leads to the conclusion that α-mannosidase is a zinc-containing metalloprotein, as observed for other α-mannosidases from various plant species [Curdel and Petek, 1980; Conti, *et al.,* 1987; Kishimoto *et al.,* 2001; Kestwal and Bhide, 2005; Blom *et al.,* 2008]. The role played by Zn^{2+} in the enzymatic reaction has not yet been elucidated. The zinc atoms may be involved in subunit interactions. Cu (II), Hg (II) and Ag (II) are potent inhibitors of some plant α-mannosidases [Woo *et al.,* 2004], whereas Mn (II), Mg (II) and Ca (II) have no effect on the α-mannosidases.

The effect of divalent cations is a useful parameter to distinguish among different α-mannosidases. Members of family 47 can be stimulated by Ca^{2+} ions whereas activity of class II α-mannosidases of family 38 exhibit diverse forms of metal ion dependency, cadmium activates the α-mannosidase activity in *T.maritima* [Nakajima, *et al.,* 2003] Co (II) is the preferred cofactor for the α-mannosidase from insect and *Bacillus sp.* [Kawar *et al.,* 2001; Nankai *et al.,* 2002], while the activity of the enzymes from jack bean require Zn (II) ions [Howard *et al.,* 1997].

The anionic detergent like SDS showed complete inhibition on the enzyme activity at 1 mM concentration that can be attributed for the partial or complete disruption of the higher order structures of the enzyme. About 73% of the original activity remained at 10 mM concentration of mannose, being a product analog it showed inhibition at higher concentration.

The purified α-mannosidase was checked for other glycosidase activities which are present in the initial crude extract with *p*NP-substrates such as *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-galactopyranoside, This α-mannosidase from *M.oleifera* seeds did not show significant activity with these substrates, this indicates that the activity of this enzyme is restricted towards *p*NP-α-D-mannopyranoside only.

Preliminary experiments were done to know the involvement of tryptophan in the active site of the enzyme as tryptophan residues are essential for substrate binding in many glycosidases, including lysozyme, glucoamylase, cellulase and xylanase. Modification of the enzyme by NBS resulted in complete loss of activity suggesting the role of tryptophan in the catalytic activity of the enzyme. When enzyme was incubated along with the substrate quenching of fluorescence is incomplete suggesting the role of tryptophan in the active site of *M.oleifera* α-mannosidase. Some studies showed that addition of a substrate like *p*NP-α-D-mannopyranoside to the enzyme prior to NBS treatment protected the enzyme [Kestwal *et al.,* 2007].

In jack bean 4 tryptophan residues are present per enzyme monomer that is involved in enzyme activity [Burrows and Rastall, 1998]. In *Canavalia ensiformis, Phaseolus vulgaris* and *Erythrina indica* seeds carboxyl and tryptophan residues present at the catalytic site are essential in enzyme activity [Paus, E. 1978; Burrows and Rastall, 1998; Kestwal *et al.,* 2007].

Plant α-mannosidases have been classified into two independently derived groups, Class I and Class II, based on inhibitor profiles [Daniel *et al.,* 1994; Eades *et al.,* 1998]. Regarding inhibitor profiles, α-mannosidases susceptible to kifunensine (KIF) and deoxymannojirimycin (DMNJ) inhibition belong to class I α-mannosidases. In contrast, those sensitive to swainsonine (SW) and 1, 4-dideoxy 1, 4-imino-D-mannitol (DIM) belong to class II mannosidases [Ahi *et al.,* 2007]. In glycosyl hydrolase classification class I α-mannosidase belongs to GH family 47 specific for α -1,2 linkage, they are activated by Ca (II) ions, whereas, class II α-mannosidase belongs to GH family 38 specific for α1,2; α1,3 ; α1,6 they are activated by Zn (II) ions.

We checked the effect of mannosidase specific inhibitors such as SW, DMNJ on the α-mannosidase activity. The activity was totally lost in presence of SW a furanose analogue at very low concentrations. While, at the same concentration DMNJ a pyranose analogue didn't show any effect.

Studies showed that at pH 5.0 the optimum pH of breadfruit α-mannosidase, SW binds strongly to the catalytic centre of the enzyme due to ionization of the SW which, in turn acts as the strong inhibitor of the enzyme [Ahi *et al.,* 2007]. In this respect the *M.oleifera* enzyme shows similar to the breadfruit α-mannosidase.

This study showed that the α-mannosidase purified from seeds of *M.oleifera* is class II α-mannosidase since it is sensitive to furanose transition state analog SW. Similar results was also observed in Jack bean, rice and bread fruit α-mannosidase [Howard *et al.,* 1997; Kishimoto *et al.,* 2001; Ahi *et al.,* 2007]. Obtaining the sequence information of this protein will give more insights into the phylogenetic relationship between different classes of α-mannosidase in a wide variety of organisms. The immunochemical results suggest *M.oleifera* α-mannosidase is immunologically similar to jack bean α-mannosidase and posses some identical antigenic determinants with that of the jack bean α-mannosidase.

The inhibition of enzyme activity caused by various synthetic glycoconjugates revealed that naphthylidene-conjugates were more potent inhibitors then the salicylidene-conjugates of the simple sugars. These results indicate that the naphthylidene-conjugates have more affinity to bind the enzyme and cause the inhibition. Similar results were reported with the Jack bean α-mannosidase [Kumar *et al.,* 2009]. It is interesting to note that the two mannosidases purified from different

plant families, legume (*Canavalia ensiformis*) and non legume (*M.oleifera*) exhibit similar sugar binding properties suggesting that they have possibly similar aminoacid sequence that permit efficient binding of bulky groups in synthetic sugars. This results states that they are highly conserved among different families of the plant kingdom.

A family of class II α-mannosidase was purified and characterized from *M.oleifera* in this present study for the first time. The enzyme was found to have heterogenous subunits of larger 66 kDa and smaller 55 kDa. Interestingly larger subunit was glycosylated with the carbohydrate content around 9.3 %. The enzyme associated with covalently bound Zn^{2+} ions. The distinctive characteristic of the *M.oleifera* α-mannosidase was its thermal stability which was not reported in other known α-mannosidase. A chemical modification study highlights the involvement of tryptophan residue at the active site of the enzyme. Mannosidase specific Inhibitor studies helped to classify this enzyme in to class II mannosidase, belonging to GH family 38. In recent years, the functional significance of the carbohydrate moieties has been increasingly appreciated. Carbohydrates covalently attached to polypeptide chains can confer many functions to the glycoprotein, for example, resistance to proteolytic degradation, the transduction of information between cells, and ligand–receptor interactions are a few to mention. Furthermore, these characteristics show the *M.oleifera* α-mannosidase importance in production of short sugar chains active biologically, and could find application in food and biotechnology industry.

CHAPTER 4B

Purification, Biochemical characterization and localization studies of acidic α-galactosidase from *Moringa oleifera* seeds

4B.1.0. Introduction

α-galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) is widely distributed in microorganisms, plants and animals [Dey and Pridham, 1972]. It is an exoglycosidase that catalyzes hydrolysis of terminal α-1-6-linked galactosyl residues from a wide range of substrates including oligo-saccharides of raffinose family sugars; raffinose, stachyose, melibiose, verbascose and polysaccharides of galactomannans, locust bean gum and guar gum. More over it also hydrolyzes glycoconjugates; glycoproteins and glycosphingolipids [McCleary and Matheson, 1974]. In plant kingdom, α-galactosidases were reported to occur in actively growing as well as in fully developed leaves and fruits, and also in seeds and tubers [Dey and del Campilo, 1984; Keller and Pharr, 1996]. The enzyme from coffee beans was one of the first α-galactosidase to be partially purified and biochemically characterized. The α-galactosidases are classified as either acid or alkaline, according to the optimal pH at which they are active. Most of the α-galactosidases isolated from plant seeds and leaves are acidic enzymes and belong to the 27 glycosyl hydrolase cluster family [Fialho *et al.,* 2008]. Some of the isoform types of the enzyme may be distributed in the cytosol as well as the vacuole, and in some reported cases also in the cell wall [Keller and Pharr, 1996]. In plants this enzyme catalyzes the hydrolysis of the free galactose from naturally occurring galactosyl-sucrose oligosaccharides such as raffinose and stachyose, as well as other α-galactosides such as galactolipids and galactoproteins. One noted function of the enzyme is in the mobilization of α-D-galactosyl residues stored within the raffinose family of oligosaccharides (RFO) and other storage polysaccharides during germination and sprouting of seeds and tubers [Soh *et al.,* 2006]. In plant species the hydrolase usually acts together with (1→4)-*β*-mannan endohydrolases (endo-*β*-mannanase) (EC 3.2.1.78) and *β*-mannosidases (EC 3.2.1.25) to degrade GMs, mainly during germination of plant seeds [Reid and Meier, 1973]. The enzyme activity has been shown to increase during seed germination concomitant with catabolism of galactomannans and other reserve polysaccharides without which this process will be restrained [Marraccini *et al.,* 2005]. Since seeds contain large amounts of such storage compounds, which are utilized as an energy source during germination, work on plant α-galactosidases has mostly focused on seed tissues [Dey and Wallenfels, 1974; Chandra Sekhar and De Mason, 1990]. More recently, site-directed mutation experiments permitted to identify amino acid residues essential for the activity of coffee bean α-Gal [Zhu *et al.,* 1995; Zhu *et al.,* 1996;

Maranville and Zhu, 2000]. It has been known that the deficiency of *α*-galactosidase leads to the Anderson– Fabry's disease in humans [Eng *et al.*, 2001]. It is an X-linked lysosomal storage problem can be treated by α-galactosidase replacement therapy [Gieselmann, 1995]. Some of the α-galactosidases isolated from various plant and microbial sources have transglycosylation activities especially at a high concentration of substrate a catalytic property that might be relevant to cell wall modification during fruit growth and development [Chin-Pin *et al.*, 2006; Gote *et al.*, 2006]. The enzyme has attracted attention in the medical application due to its capacity to remove the terminal galactose units (α 1-3 linked) from the blood group B cell surface carbohydrate moiety of glycoprotein complexes, thus generating type O red blood cells [Kruskall *et al.*, 2000; Hata and Smith, 2004]. Furthermore, α-galactosidases had many potential biotechnological applications, including: pre-treatment of animal feed to hydrolyze non-metabolizable sugars, thereby increasing the nutritive value [Ghazi *et al.*, 2003], degradation of raffinose to improve the crystallization of sucrose [Ganter *et al.*, 1988], processing of soy molasses and soybean milk [Thananunkul *et al.*, 1976], improvement of the viscosity and gelling properties of galactomannan [Dey *et al.*, 1993], stimulation of oil/gas wells through hydrolysis of the propant matrix [McCutchen, 1996], Because of their medical and technological importance, a number of α-galactosidases from eukaryotic and microbial sources have been studied.

Recently the activities of different glycosidases such as the α-galactosidase, α-mannosidase, α-glucosidase, β-glucosidase, β-galactosidase and N-acetyl β-D-hexosaminidase were checked in the extracts obtained from the seeds of *M.oleifera* that were devoid of the lectin. When analyzed using *para*-nitrophenyl derivatives of the corresponding sugars as substrates, interestingly, α-galactosidase activities were the highest followed by the α-mannosidase activity. Other glycosidase activities were found to be significantly lower compared to the α-galactosidase. Such high activity of the α-galactosidase prompted us to purify the enzyme and to study some of its properties. This is the first time that we report on the glycosidase from the seeds of *M.oleifera*. In the present study we used different chromatographic methods to purify α-galactosidase from seed kernel and looked for its biochemical characteristics. In order to identify important residues required for activity of the enzyme chemical modification studies were carried out with this enzyme. Additionally, Subcellular localization of the *M.oleifera* α-galactosidase in the seeds was studied by isolating the protein bodies from the kernel.

4B.2.0. Materials and methods

4B.2.1. Materials

Dry *Moringa oleifera* seeds (PKM_1 variety) were obtained from local market. *p*-nitrophenyl-α-D-galactopyranoside (*p*NP-α-Gal), other *p*-nitrophenyl glycosides, phenyl Sepharose CL-4B, Sephadex G-150 was obtained from Sigma-Aldrich, CM-cellulose from Whatman. Ready to use protein molecular weight marker mixture for SDS-PAGE was obtained from Fermentas. All other chemicals and reagents were of analytical grade.

4B.2.2. Assay for α-galactosidase activity

The enzyme assay and its quantification was carried out as described for α-mannosidase [Chapter 4A, Section. 2.2] except that the substrate used here was *p*-nitrophenyl-α-D-galactopyranoside. The activity of the enzyme (units/ml/min) was calculated according to the formula given below: (Enzyme activity is measured as described in chapter 4A).

4B.2.3. Extraction and purification of α-galactosidase from *Moringa oleifera*

All the purification steps were carried out at 4°C. The protein elution profile from different chromatographic columns was monitored my absorbance at A_{280}.

50 g of defatted *Moringa oleifera* seed kernels powder was kept for overnight extraction in 25 mM NaOAc buffer pH 6.0 containing 150 mM NaCl (buffer A). After extraction the sample was centrifuged (12,000 rpm for 30 min). The protein in clear supernatant (crude enzyme) was precipitated using 0-80% ammonium sulfate. The precipitate was recovered by centrifugation and dialysis against 25 mM NaOAc buffer pH 6.0 (buffer B).

Cation exchange chromatography using CM-Cellulose

Following dialysis the sample is again centrifuged to remove insoluble materials and the clear supernatant was loaded onto a Cation exchanger CM-Sepharose column (2.5 cm x 6 cm) which is previously equilibrated with 25 mM NaOAc buffer pH 6.0 (buffer B). After washing the unadsorbed proteins with the same equilibrating buffer, the bound protein was eluted with a stepwise elution with NaCl in buffer B starting from100 mM, 150 mM and 300 mM at the flow rate of 40 mL/h. 2mL fractions were collected and checked for both A_{280} and α-galactosidase activity. The fractions containing α-galactosidase activity were pooled and solid ammonium sulphate was added to 1 M concentration.

Hydrophobic interaction chromatography using Phenylsepharose CL-4B

The sample obtained above was loaded onto a phenyl-Sepharose CL -4B column (1 cm x 10 cm) which is previously equilibrated with 25 mM NaOAc buffer pH 6.0 (buffer B) containing 1 M ammonium sulphate. Unbound protein was washed with equilibration buffer and the bound proteins were eluted in absence of ammonium sulphate at the flow rate of 1 mL/min.

Gel exclusion chromatography using Sephadex G-150

The enzyme rich fractions from Phenylsepharose was concentrated using amicon concentrator and applied on to the Sephadex G-150 column (2 cm x 125 cm) previously equilibrated with 25 mM NaOAc buffer pH 6.0 containing 150 mM NaCl (buffer A). The flow rate was 15 mL/h, and the protein in the eluates was monitored at 280 nm.

4B.2.4. Native molecular mass determination

Sephadex G-150 (2 x 125 cm) was equilibrated with 25 mM sodium acetate buffer pH 6.0 containing 150 mM NaCl and 0.02% (w/v) sodium azide. The column was precalibrated using known protein standards: Phosphorylase (96 kDa), BSA (66 kDa), Ovalbumin (45 kDa), and Lysozyme (14 kDa). The apparent molecular mass of the purified α-galactosidase was estimated by passing the enzyme on this gel. The proteins were eluted with same buffer at a flow rate of 15 mL/h. The eluted protein concentration was determined by measuring the absorbance at 280 nm and the enzyme activity was monitored by standard enzyme assay.

4B.2.5. Protein and carbohydrate estimation

Protein concentrations were determined according to Bradford [1976] using BSA as standard. The total neutral sugar content of the enzyme was determined by phenol-sulphuric acid method of Dubois *et al.,* [1956] using glucose as standard. Absorbance at 280 nm was also used to estimate protein concentration in chromatographic fractions.

4B.2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out in accordance with the procedure of Laemmli [1970], using a 12% resolving gel and a 5% stacking gel under both reducing and nonreducing conditions. The samples were

cooked at 95°C for 5 min with sample buffer (4 X) mixed in 3:1 ratio. The gel was stained with Coomassie brilliant blue. The proteins are compared with Fermentas unstained protein markers as standards: (116.0 kDa) β-galactosidase, (66.2 kDa) Bovine serum albumin, (45.0 kDa) Ovalbumin, (35.0 kDa) Lactate dehydrogenase, (25.0 kDa) REase Bsp98I, (18.4 kDa) β-lactoglobulin, (14.4 kDa) Lysozyme.

4B.2.7. Optima pH and pH stability

The effect of pH on the galactosidase activity was determined by carrying enzyme assay in 100 mM of various pH buffers ranging from pH 2.0 to 9.0. The buffers used were Glycine-HCl buffer (pH 2-3), NaOAc buffer (pH 4-5), Sodium phosphate buffer (pH 6-7), Glycine-NaOH (pH 8-9). The enzyme activity was carried out at 37^{o}C. The pH stability was also carried using the same buffers where the enzyme is pre-incubated for 24 h at 37^{o}C and the residual activity of the liquated enzyme was assayed using standard enzyme assay.

4B.2.8. Temperature optima and stability

Determination of optimum temperature for the α-galactosidase was performed with *p*-nitrophenyl-α-D-galactopyranoside (5 mM) in 100 mM NaOAc pH 5.0 using incubation temperatures in the range of 30 to 80°C. Thermal stability was investigated after incubation of the enzyme at again 30 to 80°C for 1 h an aliquot was drawn and immediately cooled. The residual enzyme activity was determined by standard assay method. The activity of the untreated enzyme was used as the control (100%).

4B.2.9. Effect of various metal ions, sugars and inhibitor reagents

The effect of various metal ions, simple sugars, reducing agents and inhibitor reagents on the enzyme activity was tested by the standard assay with enzyme samples pre-incubated with each of the compounds tested for 30 min at 37°C.

4B.2.10. Substrate specificity and kinetic studies

The relative substrate specificity of α-galactosidase towards various synthetic glycosides was determined at 5.0 mM of substrate concentrations. The Michaelis–Menton kinetic parameters (K_M and V_{max}) were determined by incubating the enzyme at optimum temperature/pH with different concentrations of substrate.

4B.2.11. Chemical modification studies

Chemical modification studies of the purified α-galactosidase were done using NBS in acetate buffer (20 mM, pH 5.0), PMSF in methanol and DEPC in phosphate buffer (20 mM, pH 6.0) which are, tryptophan, serine and histidine modifying agent, respectively. Purified enzyme 0.5 mL was incubated with 0.5 mL modifying agent (20

mM), so as to get a final concentration of the modifying agent as 10 mM, for 30 min, followed by estimation of residual enzyme activity.

4B.2.12. Isolation of protein bodies from the seeds of *M.oleifera*

M.oleifera seeds were soaked overnight, placed on a wet filter paper. After soaking the seed coat is separated from the kernel. The fresh kernel was then chopped with a razor blade into small pieces. The sliced kernel pieces were homogenized using a mortar and pestle in 20 mM HEPES buffer pH 7.4, containing 250 mM Sucrose, 2 mM $MgCl_2$, 1 mM EGTA and 1 mM EDTA. The homogenate was then filtered through a fine cloth. The cell walls and debris were removed by centrifugation at 1,000 x g for 5 min and the supernatants used for the isolation of protein bodies. The supernatants were layered onto a sucrose density gradient solution from 10-65% sucrose as shown in the figure [4B.6-A] and centrifuged at 60,000 x g for 2 h. Distinct bands formed after sucrose density gradient centrifugation were carefully aspirated and visualized microscopically. The suspension containing the protein bodies was washed with 20 mM HEPES buffer pH 7.4, sonicated and centrifuged. The supernatant was analyzed for the α-galactosidase activity and proteins identified by 12% SDS-PAGE.

4B.3.0. Results

4B.3.1. *M.oleifera* α-galactosidase purification

The defatted *M.oleifera* seed kernel when extracted in the acidic buffer like sodium acetate pH 6.0 and when analyzed for the glycosidase activities contained significant amount of α-galactosidase addition to other glycosidases.

In CM-cellulose column, almost all the enzyme activity was retained on the column. The elution pattern with NaCl showed two peaks. The first peak eluted in 100 mM NaCl was devoid of α-galactosidase activity. The second peak eluted with 150 mM NaCl contained the enzyme [Figure: 4B.1-A]. The pooled and concentrated eluate from CM-cellulose was applied on phenyl Sepharose gel at high ammonium sulphate concentration. The enzyme specifically bound on this hydrophobic gel and could be easily desorbed using buffer without salt [Figure: 4B.1-B]. To further separate the enzyme from other contaminating proteins and to determine its molecular mass the eluates were pooled, concentrated and were subjected to gel filtration on G-150. The protein was eluted as a single peak with apparent molecular mass was 66 kDa [Figure: 4B.2 A & B]. α-galactosidase was purified from *M.oleifera* seeds using a combination of several chromatographic steps, the results were summarized in Table: 4B.1. Around 29.6 fold purification was obtained with total yield of approximately 10.5%.

When this sample was analysed using SDS-PAGE it gave a single protein band at approximately 66 kDa, under both reducing and non-reducing conditions confirming that the isolated α-galactosidase is a monomeric enzyme consists of single polypeptide chain [Figure: 4B.3]. The purified enzyme did not contain any covalently bound carbohydrate as evidenced by phenol-sulphuric acid method.

4B.3.2. Biochemical characterization of *M.oleifera* α-galactosidase

The optimum pH of *M.oleifera* α-galactosidase activity was determined to be around pH 5.0 at 37°C, the enzyme exhibited maximum activity (more than 70%) in the pH range of 4.0 to 6.0, only 43% and 10% of the activity remained at pH 7.0 and 8.0 respectively. The pH optimum after incubating the enzyme in different pH buffers indicates that the enzyme was more stable in the acidic environment and lost its activity gradually as the pH increases to the basic side. Only 24% of the activity was retained at pH 8.0 after incubating for 24 h [Figure: 4B. 4-A]. The activity is irreversibly lost at neutral pH. The effect of temperature was studied in the range of

30-80°C, α-galactosidase activity increases with temperature up to 50°C after which it decreased. On reaching 80°C only 10% of the activity remained [Figure: 4B.4-B]. The thermostability of the enzyme was determined by preincubating the enzyme at various temperatures ranging from 30 to 80°C for 30 min. More than 80% of the activity was seen at 50°C after 1 h of incubation.

The effect of various chemical agents and metal ions was tested against the *M.oleifera* α-galactosidase activity is shown in Table: 4B.2. Of all the divalent metals tested Hg^{2+}, Ag^{2+}, Cu^{2+} and denaturant like SDS showed potent α-galactosidase inhibition at the concentration of 1 mM. On the contrary other metal ions such as Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+} and Ni^{2+} didn't show any significant reduction of enzyme activity at 1 mM concentration. Among various sugars tested galactose showed 60% inhibition at 10 mM concentration. Guanidine hydrochloride did not show any effect and urea slightly enhanced the activity possibly due to the exposure of the catalytic site of the enzyme to the substrate. The enzyme did not exhibit any hemagglutinating property.

4B.3.3. Substrate specificity and kinetic studies

Results of the substrate specificity towards synthetic substrates are summarized in Table: 4B.3. The K_m and V_{max} value was measured using *p*-nitrophenyl-α-D-galactopyranoside and was found to be 0.49 mM and 0.88 U/mg respectively.

4B.3.4. Chemical modification studies

Chemical modification studies were carried out to get information about amino acid residues involved in the active site of the enzyme. The involvement of tryptophan and histidine in the catalytic activity is evident by the modification of these residues, which resulted in significant loss of activity by NBS and DEPC treatment. Around 94% and 68% decrease in the enzyme activity was observed with NBS and DEPC treatment, respectively. No loss of activity was observed in presence of PMSF [Figure: 4B.5]

4B.3.5. Detection of α-galactosidase activity in protein bodies

In order to identify the localization of this acidic hydrolase we purified, we isolated the protein bodies from the seeds of *M.oleifera* by sucrose density gradient centrifugation as described under methods [Figure: 4B.6-A]. Several band regions were identified after the centrifugation and the band region corresponding to the 45% sucrose contained largely intact protein bodies when observed microscopically

[Figure: 4B.6-B]. These were further processed to analyze the enzyme activity and the protein composition. The α-galactosidase activity was mainly concentrated in the soluble fraction of the protein bodies together with other storage proteins [Figure: 4B.6-C].

FIGURES

Figure: 4B.1 A & B

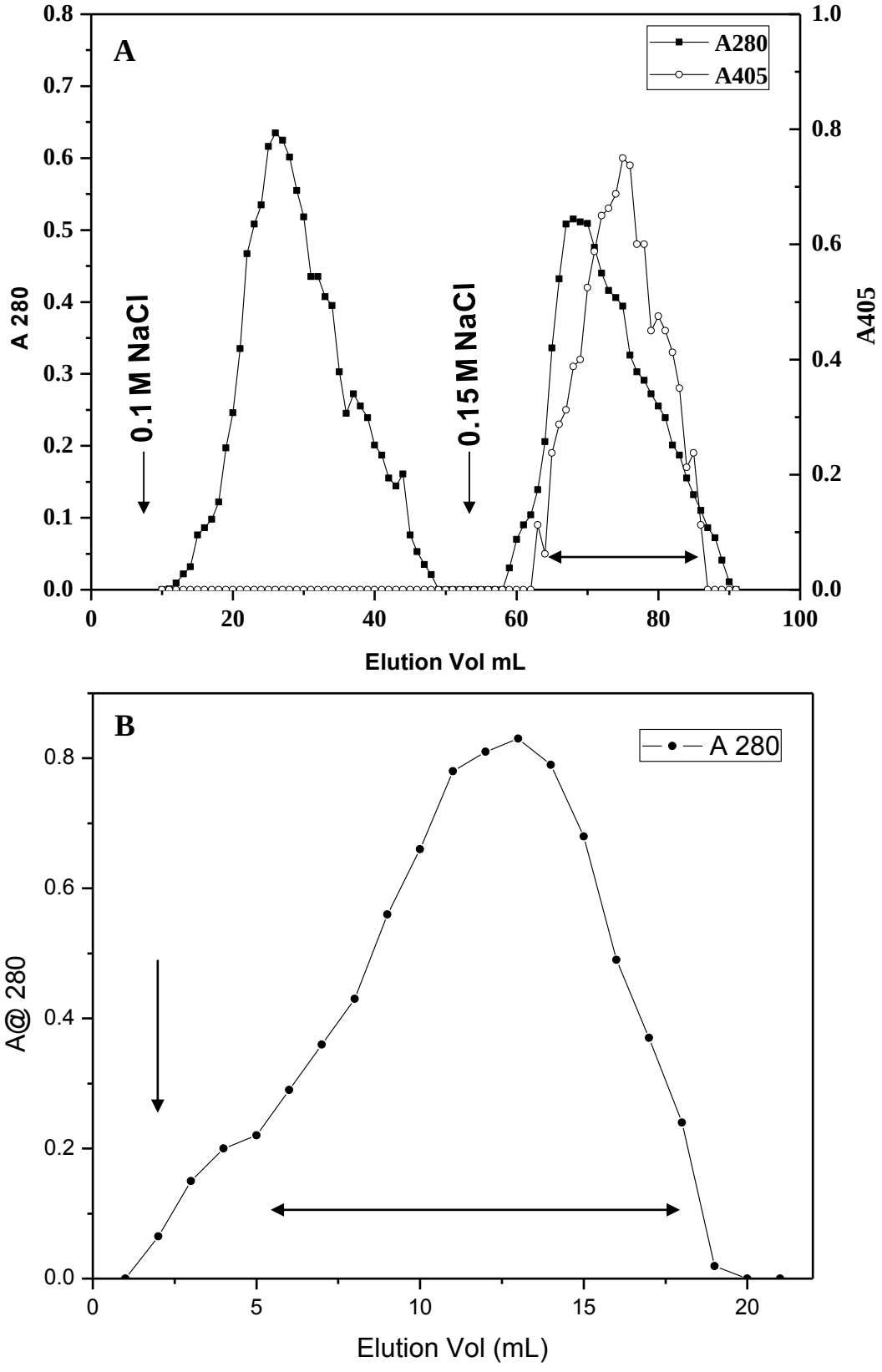
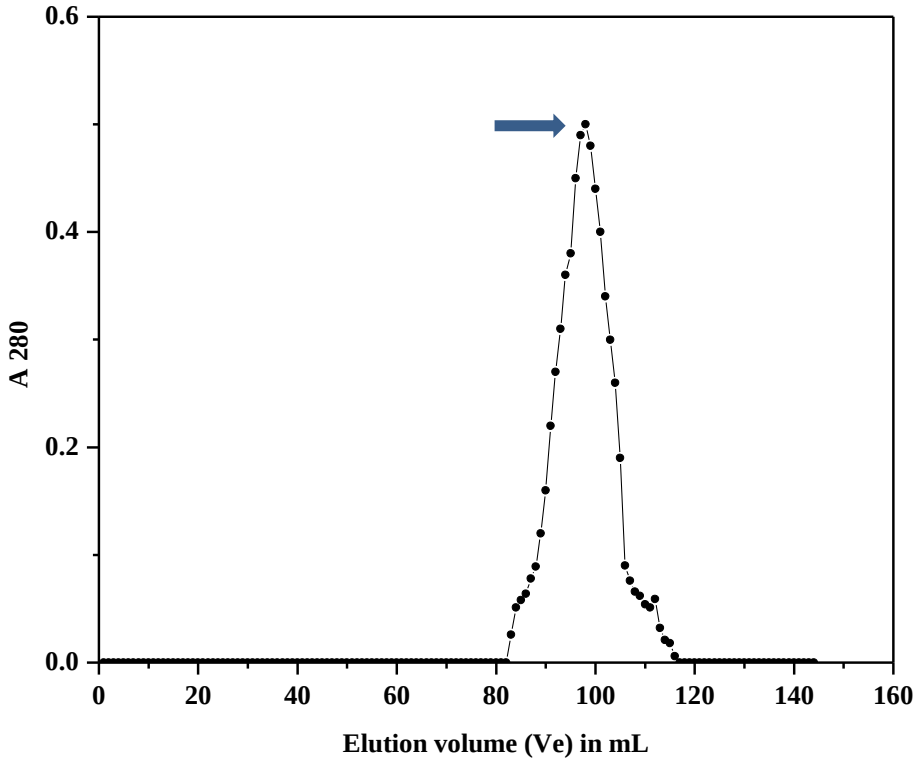
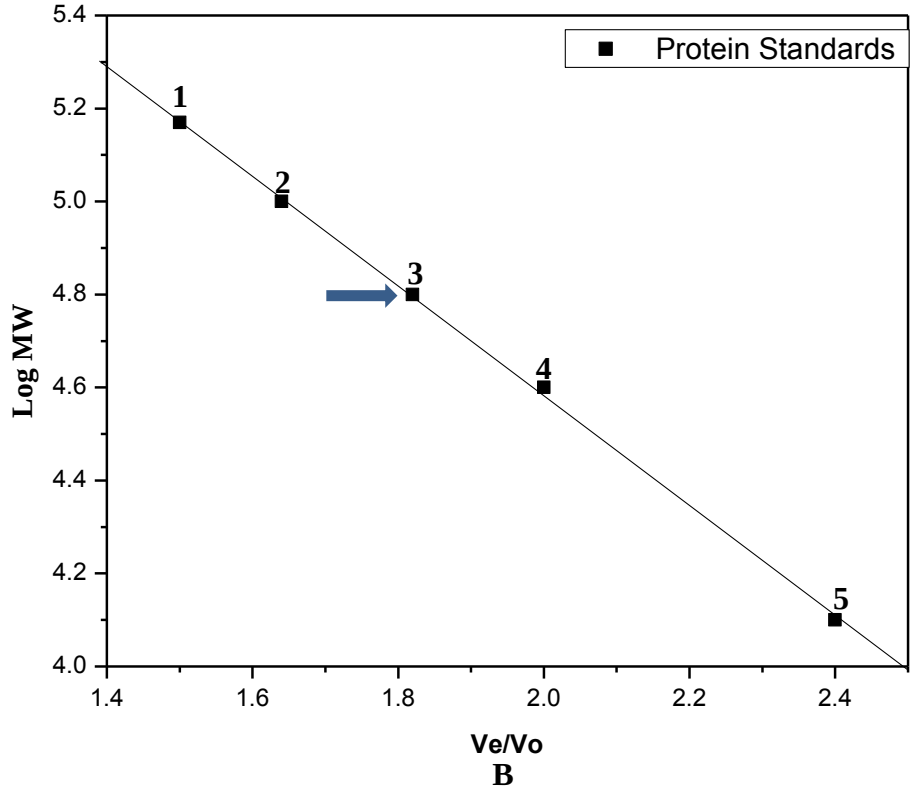


Figure: 4B. 1 A & B. (A) CM-cellulose chromatography: The ammonium sulphate precipitated proteins from the crude extract after dialysis against 25 mM NaOAc buffer pH 6.0 was applied on to CM-cellulose gel (2.5 x 6 cm) pre equilibrated with 25 mM NaOAc buffer pH 6.0. After washing extensively with the equilibration buffer, elution was carried out with one bed volume of 100 mM NaCl followed by 150 mM NaCl. Arrows indicates point of application of NaCl. Bar indicates the enzyme active fractions pooled **(B) Elution profile of *Moringa oleifera* α-galactosidase from phenyl Sepharose:** Enzyme rich fractions from CM–Cellulose chromatography were concentrated and made to 1M ammonium sulphate and applied on a phenyl Sepharose gel (1 x 10 cm) equilibrated with 25 mM NaOAc buffer pH 6.0 containing 1 M ammonium sulphate. After washing the gel with this buffer, bound protein was eluted using 25 mM NaOAc buffer pH 6.0, without salt. 1 mL fractions were collected and protein monitored at 280 nm and assayed for α-galactosidase activity. Bar indicate enzyme active fractions.

Figure: 4B. 2 A & B.



A



B

Figure: 4B.2 A & B: Gel filtration and Molecular weight determination of the α-galactosidase: (A) The phenyl Sepharose eluates containing α-galactosidase were pooled, concentrated and applied on Sephadex G-150 (2 x 125 cm) gel filtration column equilibrated with 25 mM NaOAc buffer (pH 6.0) containing 0.15 M NaCl . 1.0mL fractions were collected. **(B)** Standard graph of known molecular weight proteins: 1. Alcohol dehydrogenase 150 kDa. 2. β-galactosidase 116 kDa. 3. BSA 66 kDa. 4. Ovalbumin 45 kDa and 5. Lysozyme 14 kDa. Arrow indicates point where α-galactosidase was eluted.

Figure: 4B.3

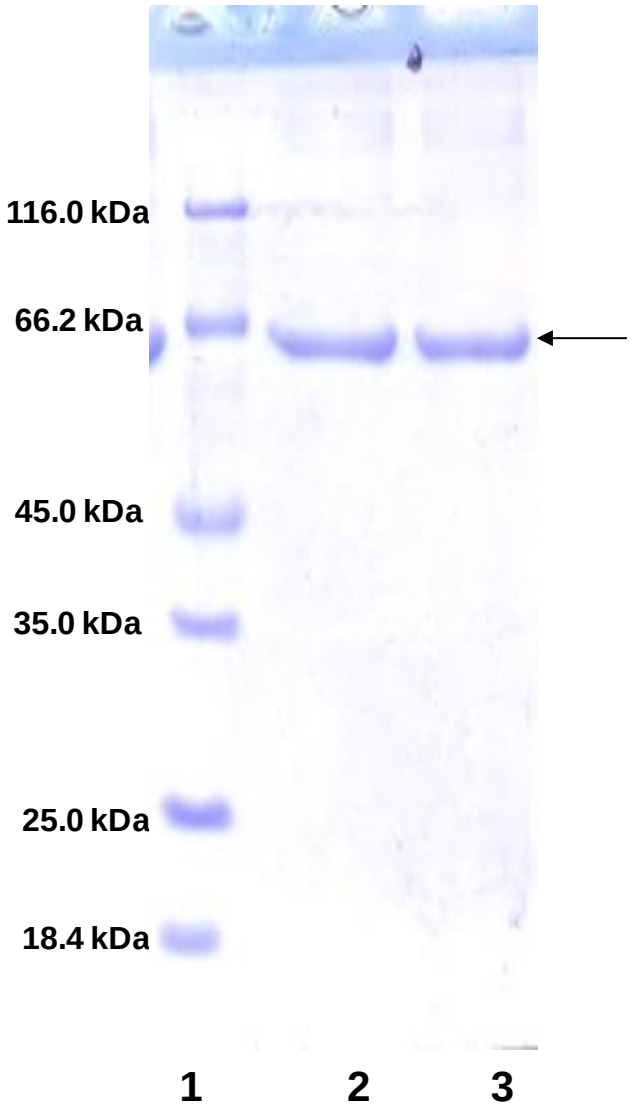


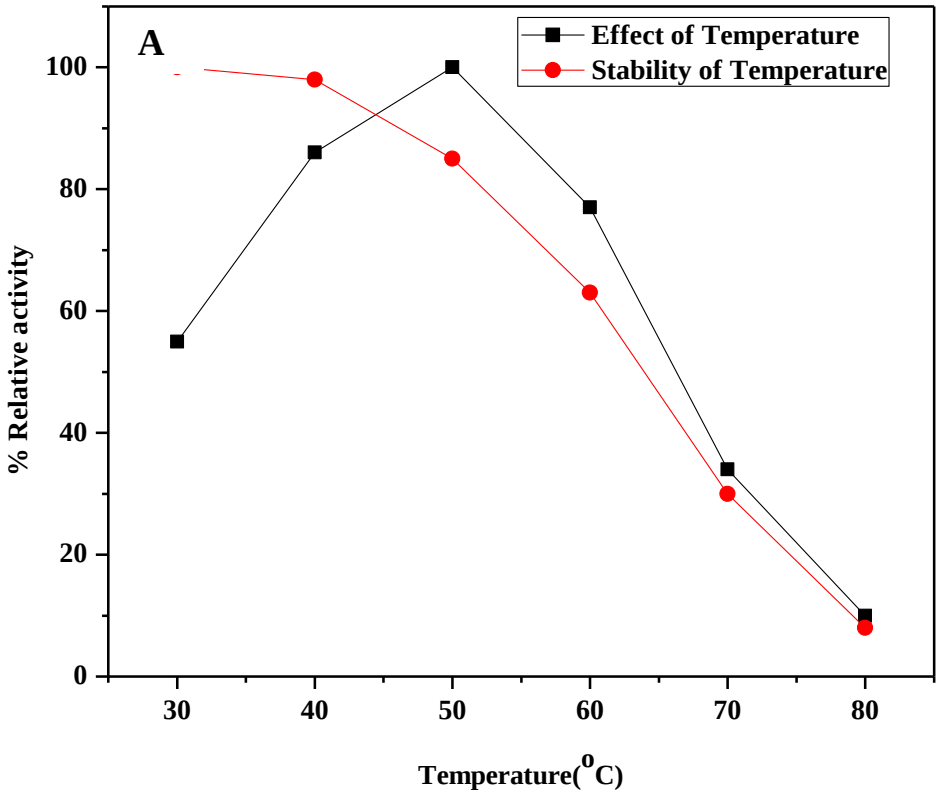
Figure: 4B.3: 12% SDS-PAGE analysis of the purified α-D-galactosidase:

Lane 1. Molecular weight markers (Fermentas),

Lane 2 and 3, Purified α-galactosidase sample after gel filtration under reducing and non-reducing conditions respectively.

Arrows corresponds to the α-galactosidase band**.**

Figure: 4B. 4 A & B



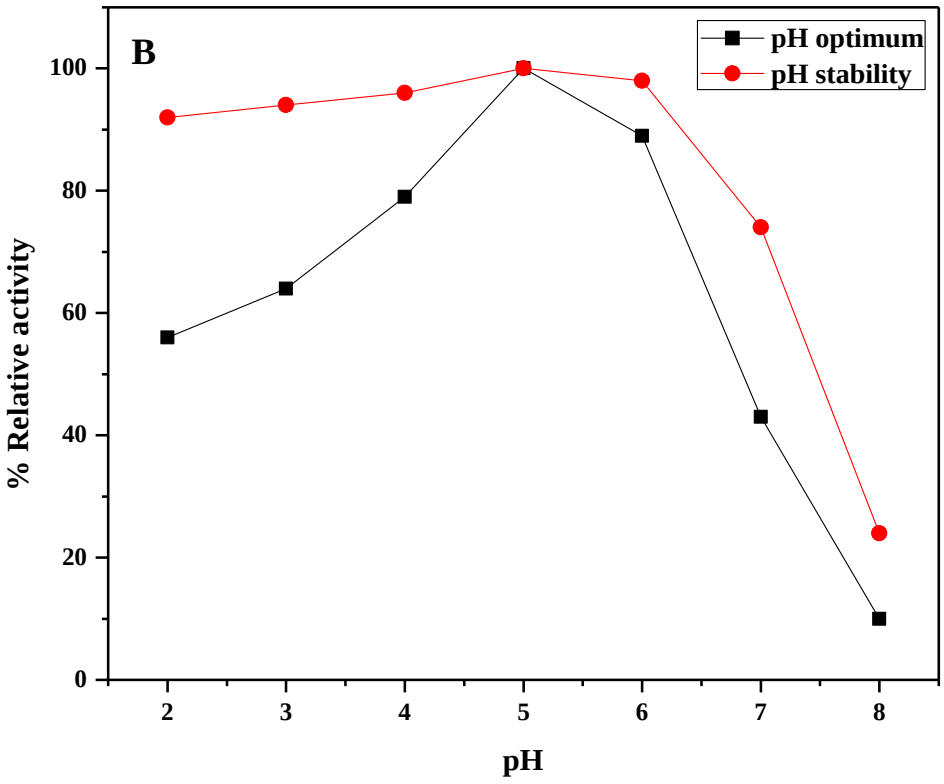


Figure: 4B.4 A & B.

(A) Effect of pH and pH stability: Effect of pH on enzyme activity was carried out by incubating the enzyme with different buffers (pH ranging from pH 2-8) and the pH stability of the enzyme was determined by pre-incubating the enzyme at same pH for 24 h, the residual activity was determined using standard assay. Relative activity was calculated in relation to pH giving highest activity considered as 100%.

(B) Effect of temperature and Temperature stability: The enzyme activity was carried out by incubating the enzyme at different temperatures ranging from 30-80°C. The stability of the enzyme was determined by incubating the enzyme at various temperatures ranging from 30-80°C for 1 h and the residual activity was determined using standard assay. Relative activity was calculated in relation to temperature giving highest activity considered as 100%.

Figure: 4B.5

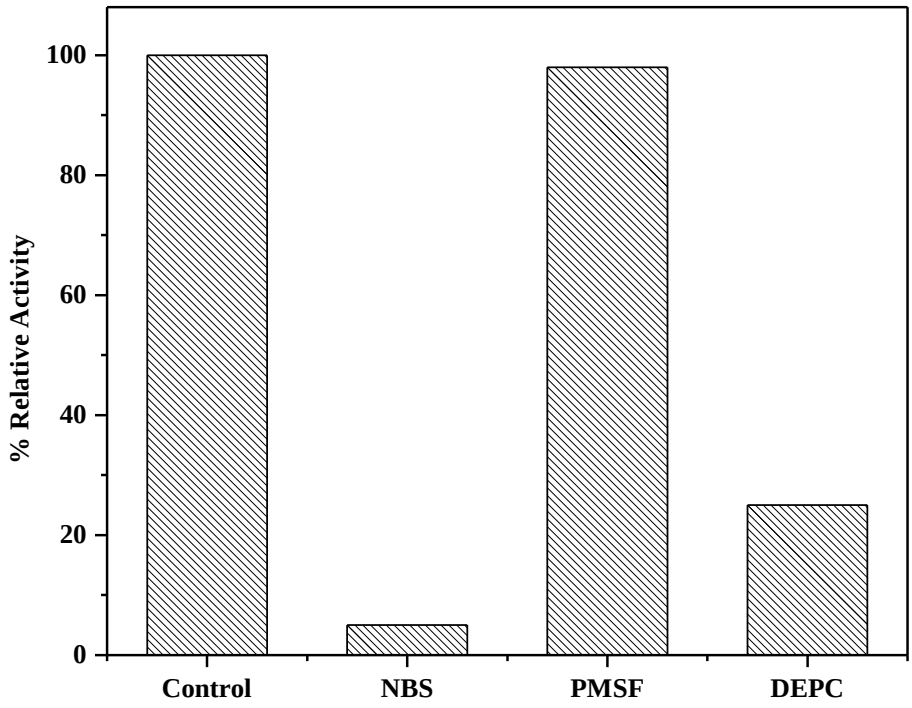


Figure: 4B.5. Chemical modification studies. Purified *Moringa oleifera* α-galactosidase enzyme was incubated with modifying agent, so as to get a final concentration of the modifying agent as 10 mM for 30 min and the residual activity was later assayed using standard assay condition. Sample without modification is taken as 100% (Control).

Figure: 4B.6A, B & C.

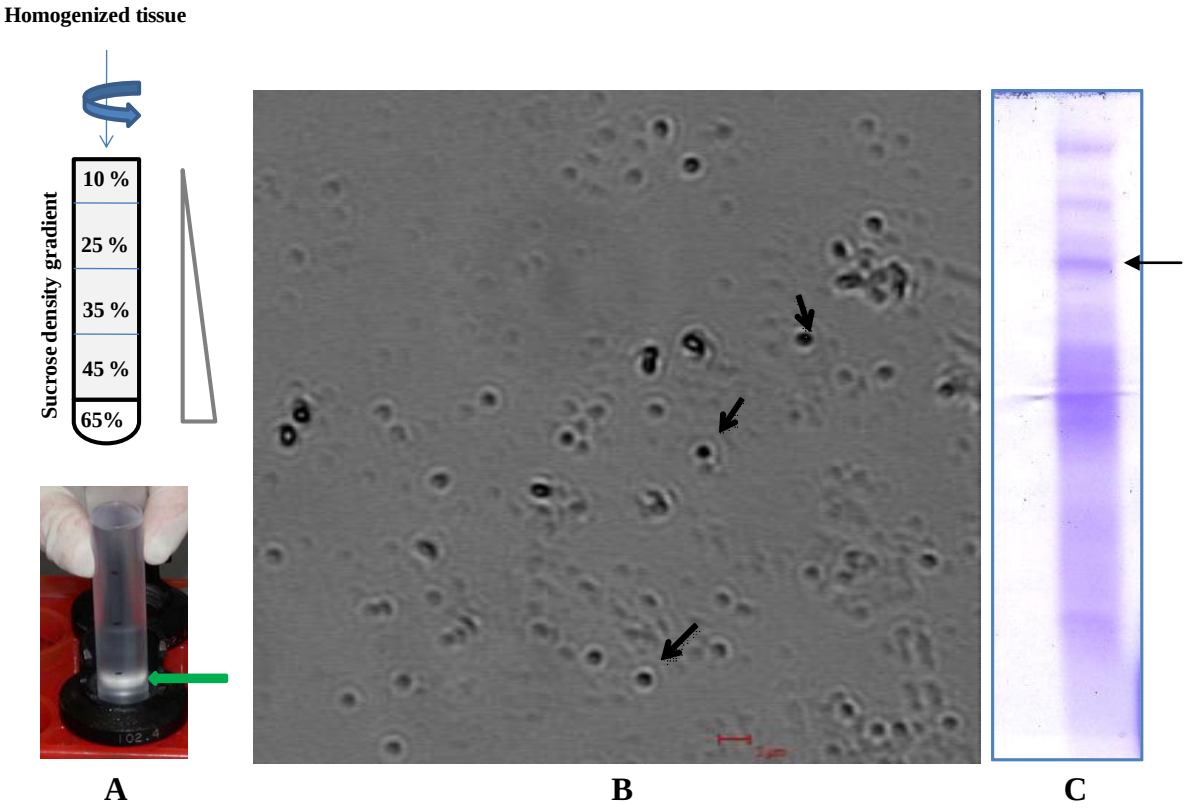


Figure: 4B.6 A, B & C. Isolation of protein bodies: (A) Schematic representation of the sucrose density gradient centrifugation carried out in this experiment, **(B)** Micrograph picture of Protein bodies and **(C)** 12% SDS-PAGE of the proteins extracted from the protein bodies. **(A)** The overnight soaked seed kernel were homogenized in the fractionation buffer, filtered and layered on to the sucrose density gradient. Centrifuged at 60,000 x g for 2 h. Distinct layers formed after centrifugation was carefully aspirated and checked for the enzyme activity. Arrow indicates the layer which has α-D-galactosidase activity. **(B)** The protein bodies isolated from the seed kernel of *Moringa oleifera* using sucrose gradient density centrifugation was visualized using confocal microscope transmission image. Arrow indicates protein bodies. **(C)** The proteins from the protein bodies were extracted by sonicating and thus extracted protein were passed through CM-cellulose column and the bound protein was eluted with 150 mM NaCl. The eluted protein was analyzed using 12 % SDS-PAGE. Arrow indicating the possible α-galactosidase protein.

TABLES

Table: 4B.1. Purification of α-galactosidase from *Moringa oleifera* [a]

Purification stage	Total protein (mg)	Total Activity (units)[b]	Specific Activity [c]	Purification fold	Yield
Crude extract [a]	1400	840	0.6	1	100
CM-cellulose	260	423	1.6	2.6	50.3
Phenyl Sepharose CL-4B	20	136	6.8	11.3	16.1
Sephadex G-150	5	89	17.8	29.6	10.5

[a] 50 g of defatted *Moringa oleifera* seed kernel powder was used for the purification in different chromatographic steps. Proteins were measured in chromatographic fractions at 280 nm.

[b] One unit of enzyme activity was defined as amount of enzyme hydrolyzing 1 µmol of *p*-nitrophenol from *p*-nitrophenyl-α-D-galactopyranoside per min at pH 5.0 and 37°C.

[c] The specific activity of the enzyme is expressed as units per mg protein. (U/mg of protein).

Table: 4B. 2. Effect of chemical agents, sugars and divalent metal ions on α-galactosidase activity.

Effector agent [a]	Relative galactosidase activity (%)[b]
Control	100
EDTA	94
β-mercaptoethanol	95
Iodoacetamide	89
D-Galactose	40
D-Mannose	91
D-Glucose	95
SDS	ND
Mg^{2+}	98
Mn^{2+}	94
Cu^{2+}	ND
Ca^{2+}	90
Hg^{2+}	ND
Fe^{2+}	96
Zn^{2+}	89
Fe^{2+}	94
Ag^{2+}	ND

[a] Sugars and EDTA was 10 mM. Concentration of β-mercaptoethanol was 1% (v/v). All the concentrations of the metal ions and chemical reagent was 1mM.

[b] Relative activities were calculated in relation to control without any addition of effector, this was considered as 100%. ND. Not Detected.

Table: 4B. 3. Relative substrate specificity of purified α-galactosidase from *M.oleifera.*

Substrates [a]	**Relative activity (%)** [b]
pNP-α-D-galactopyranoside	100
pNP-β-D-galactopyranoside	12
pNP-α-D-mannopyranoside	2
pNP-α-D-glucopyranoside	20

[a] Enzyme activity was determined using synthetic substrate as described in section 4B.2.2. at 37°C .

[b] The activities are expressed as percentage of the activity calculated with p-nitrophenyl α-D-galactopyranoside which is taken as 100%

4B.4.0. Discussion

We studied some enzymatic properties of α-galactosidase from *M.oleifera.* The molecular weight of the purified enzyme was about 66 kDa as determined by both gel filtration and SDS-PAGE analysis, suggesting a monomeric form. Other plant α-galactosidases, *Vitis vinifera* [Kang and Lee, 2001], *Oryza sativa* [Kima *et al.,* 2002] and *Helianthus annuus* [Kim *et al.,* 2003] have molecular masses in the range 40-50 kDa. The pH optimum of the enzyme was around 5.0 when *p*-nitrophenyl α-D-galactopyranoside (*p*NP-α-Gal) was used as a substrate. The decline in activity of the enzyme beyond pH 6.0 and exhibiting optimal activity at pH 5.0 suggests this enzyme to be acidic nature. Similar findings are reported in other plant galactosidases [Gao and Schaffer, 1999]. Most of the plant α-galactosidases are thermally stable and the *M.oleifera* was also stable. However, α-galactosidases from microbial sources exhibit more thermal stability. Reducing agents like β-mercaptoethanol and iodoacetamide did not affect the enzyme activity significantly suggesting non involvement of sulfhydryl groups in the activity. Absence of inhibition in presence of 10 mM EDTA suggests that this enzyme is not a metalloenzyme. This is in agreement with the other α-galactosidase [Viana *et al.,* 2005]. The effect seen with the SDS could possibly be due to the loss of enzyme conformation as has been reported for other enzymes. The inhibition of α-galactosidase with Hg^{2+} and Cu^{2+} ions is usually attributed to its reaction with thiol, carboxyl, amino and imidazolium groups, whereas inhibition with Ag^{2+} may be attributed to the reaction with only carboxyl and or histidine residues at the active site of α-galactosidase. Most of the metal ions tested for the effect on catalytic activity of α-galactosidase do not appear to have stabilizing/activating effect. Similar effects were seen with the enzymes isolated from other sources such as the *T. multijuga* seeds [Fialho *et al.,* 2008], water melon seeds [Itoh *et al.,* 1986] and soya bean seeds [Viana *et al.,* 2005]. Additionally, it is known that the *Oryza sativa* enzyme also lost its activity in presence of these metal ions and it is suggested that this is due to its binding at the catalytic site preventing the substrate from binding to the enzyme [Li *et al.,* 2007]. The inhibition of α-galactosidases by various sugars and their derivatives has also been reported [Chinen *et al.,* 1981]. Generally D-galactose is a competitive inhibitor of many α-galactosidases [Agnantiari *et al.,* 1991; Gao and Schaffer, 1999], which may be due to the fact that this sugar is a substrate analogue.

α-galactosidase from *Glycine max* and other legume are associated with hemagglutinin properties [Hankins *et al.*, 1980]. Previous study on the lectin form the same source [Tejavath *et al.*, 2011] it was described that the interaction of the glycosidases with that of lectin affigel under acidic conditions. Further, in the initial purification steps of MoSL lectin using galactose column, α-galactosidase was appeared as a minor contaminant, owing to support the concept of MoSL interactions with α-galactosidase from the same source. They might co-localized in the same compartment. Earlier reports on plant α-galactosidase showed that tryptophan, and histidine is important amino acids present at the catalytic site of this enzyme [Zhu *et al.*, 1995; Zhu *et al.*, 1996; Maranville and Zhu, 2000]. To cross check if it is true for the *M.oleifera* α-galactosidase, amino acid specific chemical modification studies were carried out with this enzyme. It was observed the both NBS and DEPC contributed to the loss of enzyme activity suggesting their role of tryptophan and histidine respectively in the enzyme activity.

Though the α-galactosidase has been reported from some plant seeds, little information are known about its specific localization in the seeds. Among the legumes, the plant glycosidases together with the seed storage proteins and lectins have been shown to be present in protein bodies of the seeds suggesting possible *in vivo* interactions among these different proteins that may be of physiological significance during seed growth and development.

The specific localization of the enzyme to the protein bodies strongly supports the possible *in vivo* function of this enzyme during the seed growth and development in removing galactose residues from protein/oligosaccharides. The α-galactosidase synthesized during the seed development is possibly targeted to protein bodies. Natural substrates for this enzyme such as the raffinose oligosaccharides, and galactosyl cyclitols accumulate mainly in the cytosol. During germination, there is considerable swelling of the protein bodies to form a vacuole, where in the enzyme can preferentially act on the natural substrates and aid in their hydrolysis for effective utilization of the products by the growing seedling [Viana *et al.*, 2005]. In the present study, identification of the protein bodies and the enzyme as one of the constituent proteins of the protein bodies, strongly suggests the *in vivo* function of this enzyme during seed germination.

In conclusion, we have purified α-galactosidase from *M.oleifera*, and studied various properties of the enzyme, which show both similarities and differences from other α-galactosidases reported earlier. Future studies on the enzyme to determine its complete sequence by proteomics approach or by molecular cloning should reveal the relatedness of the *M.oleifera* enzyme to other enzymes.

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PUBLICATIONS

Understanding the roles of proteins in plant seeds with gel electrophoresis

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Moringa oleifera (MO) seeds are both nutritionally and medically important, but as yet, are not well understood and so potentially underexploited. The seeds contain a water-soluble coagulant protein (MOCP), which we have purified from soluble extracts using ion exchange and size exclusion chromatography. The purified protein exhibited a molecular mass of 13 kDa. In SDS-PAGE and tricine-PAGE, under reducing conditions it gives a molecular mass of 6.5 kDa, suggesting it to be a homodimer. It is also useful to immuno-purify the seed MOCP, and to study its localization in seeds. Thermal denaturation studies show that MOCP is structurally stable and unfolding is completely reversible.

Isolation of a Galactose Specific Lectin from *Moringa Oleifera* Seeds: Affinity Purification and Characterization

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A galactose-specific lectin was isolated from the crude extracts of the *Moringa oleifera* seed powder using a combination of ammonium sulphate fractionation (0-75%), Sepharose-galactose affinity chromatography and gel filtration on Sephadex G-150. *Moringa oleifera* seed lectin agglutinates pronase treated rabbit erythrocytes. It is a non-glycoprotein whose activity is inhibited by galactose and its derivatives. Additionally, sugar inhibition studies using synthetic sugars revealed some insights of the lectin properties. Galactosyl Naphthyl Imine, a galactose derivative was found to be a most potent inhibitor for Hemagglutinating activity (MIC= 0.04 mM). The native molecular mass of the purified lectin by MALDI-TOF analysis and gel filtration was found to be 25.8 kDa and 27.0 kDa, respectively. By SDS-PAGE analysis under reducing conditions, the protein gave a single band (Mr 27kDa) suggesting it to be a monomer. Interestingly the protein cross-reacts with an antibody to a galactose specific legume lectin (Dolichos lablab 31 kDa subunit antibody) suggesting some antigenic similarities among this non-legume and legume lectin, which share similar sugar specificity.

Introduction

Lectins, by virtue of their exquisite sugar specificities and cell-agglutinating properties, have been found as useful tools in widespread applications for monitoring the expression of cell-surface carbohydrates as well as for the purification and characterization of glycoconjugates [1–3]. Extensive study of sequence homology and 3D structure of various plant lectins suggests that they are conserved throughout evolution and thus may play, yet unknown, important physiological roles [4, 5]. Seeds of legumes such as *Dolichos lablab* (Indian lablab beans), peas and beans have long been known to represent a rich source of lectins [6-8] in view of their high potential as tools in biochemical and biomedical research, both for preparative and for analytical purposes [9]. We are looking for new lectins in the tropical and subtropical flora that might have special properties in terms of sugar-binding, specificity and / or stability. *Moringa oleifera* (MO) (family; Moringaceae) is a tropical plant having many medicinal values which is largely grown in India [10]. Here we describe the affinity purification of a galactose specific lectin from the seeds of deshelled MO. The seeds contain high amounts of coagulant protein, which has been used to purify water and has also been characterized in our laboratory [11, 12]. There are reports on the isolation and identification of lectins from the MO seeds which differ in their properties from the galactose specific lectin we purified in this study [13-15].

The present work deals with the preparation of affinity matrix (Serlaose-divinyl sulfone-galactose) which was successfully used by us for the purification of galactose-specific lectins from *Dolichos lablab*