# STUDIES ON THE MODULATORY ASPECTS OF CERTAIN DIETARY LECTINS IN RELATION TO HYPERSENSITIVITY AND IMMUNOGENICITY

*A Thesis submitted to the*

**UNIVERSITY OF MYSORE**

*For the award of the degree of*

*Doctor of Philosophy*

*In*

**BIOCHEMISTRY**

*By*

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**May 2007**

# DECLARATION

I hereby declare that the thesis entitled **"STUDIES ON THE MODULATORY ASPECTS OF CERTAIN DIETARY LECTINS IN RELATION TO HYPERSENSITIVITY AND IMMUNOGENICITY"** submitted to the **University of Mysore** for the award of degree of **Doctor of Philosophy** in **Biochemistry,** is the result of research work carried out by me under the guidance of **Dr. Y. P. Venkatesh,** Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute (CFTRI), Mysore – 570 020, India, during the period of July 2004 to May 2007. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

Date: May 2007

Place: Mysore **(S. N. PRAMOD)**

**Dr. Y. P. Venkatesh, PhD**
Scientist,
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# CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON THE MODULATORY ASPECTS OF CERTAIN DIETARY LECTINS IN RELATION TO HYPERSENSITIVITY AND IMMUNOGENICITY"** submitted by **Mr. S. N. Pramod,** for the award of **Doctor of Philosophy** in **Biochemistry** to the **University of Mysore** is the result of research work carried out by him in the Department of Biochemistry and Nutrition, under my guidance during the period July 2004 to May 2007.

Date: May 2007

Place: Mysore

**(Y. P. VENKATESH)**
Guide

*Dedicated to……*

*My Father, Late S. K. Narayana*

## ACKNOWLEDGEMENTS

*It is a feeling of justifiable pleasure that I wish to express my deep sense of gratitude and indebtness to my research mentor Dr. Y. P. Venkatesh for suggesting the thesis topic and for his valuable, inspiring guidance, constant encouragement and affectionate concern throughout my research work. I will always cherish the academic freedom and friendly atmosphere that I enjoyed in his laboratory.*

*I wish to express my sincere thanks to Dr. V. Prakash, Director, CFTRI for providing me an opportunity to work in this institute. I am also thankful for his keen interest and encouragement during this research work. I am also thankful to the previous acting director Dr. K. N. Gurudutt for his valuable advice, encouragement and interest in this study.*

*I am grateful to Dr. P. V. Salimath, Head, Department of Biochemistry and Nutrition, CFTRI for providing the departmental facilities, and for his constant help and encouragement throughout this research work. Special thanks to Dr. S. G. Bhat, former head, Department of Biochemistry and Nutrition, CFTRI for his valuable suggestions and help during my research work.*

*It would not have been possible to carry out this work without the invaluable cooperation from the patients and volunteers. My heartfelt thanks to each one of them. The clinical support rendered by Dr. P. A. Mahesh and Dr. Amrutha of Allergy, Asthma and Chest Centre Mysore, as well as Dr. B. N. Ramesh, Purnima Nagaraj, and staff of Health Centre, CFTRI are gratefully acknowledged.*

*I am highly indebted to Dr. S. P. Muthukumar for his invaluable help and co-operation in conducting the animal experiments. I am grateful to Dr. P. Saibaba and the members of Institutional Animal House Facility, CFTRI for providing excellent support. I would also like to thank the staff of Central Instruments and Facilities Services (CIFS) for all their help and assistance.*

*I am extremely thankful to all teachers and staff of our department for making my stay here a fruitful and memorable experience. Special thanks to Dr. T. P. Krishnakantha, and Dr. K. S. Jagannatha Rao for their help and support. Special thanks, to Mr. S. Vishwanatha for his help in drawing blood from volunteers. I would like to thank Mr. Vijay kumar for his help at office work.*

*I appreciate the help and support of my senior Venkatesh Hegde, Krithika, my labmates, Sreenath, Harish Babu, Fatima Clement, Chandrashekar, Santhosh and Kiran*

*kumar. I extend my warm gratitude to all my friends and well wishers in the department and in the institute. My special thanks to all of them for all the help and concern. I would also like to thank all my classmates and friends outside the institute for their help and support during my stay in Mysore.*

*I am privileged to have my family sharing my dreams and supportive throughout. My beloved mother Nagarathna and brother Harish are always a source of inspiration and moral support to me, ultimate kudos to them. I would also thank all my family members and relatives for their timely help and constant encouragement.*

*Finally, I gratefully acknowledge the financial assistance in the form of senior research fellowship (SRF) from the Council of Scientific and Industrial Research (CSIR), New Delhi.*

***S. N. Pramod***

# ABSTRACT OF THE THESIS

Lectins are carbohydrate binding-proteins or glycoproteins of non-immune origin. Adverse reactions caused by the activation of mast cells/basophils by lectins present in food sources are generally included under 'non-allergic food hypersensitivity', commonly known as false food allergy. Dietary lectins can either interact with carbohydrates on cell-bound IgE or directly with the carbohydrates of cell surface glycoproteins/glycolipids on basophils or mast cells resulting in non-allergic food hypersensitivity reactions. Lectins may modulate the immune system in various ways. Dietary lectins can lead to the generation of an anti-lectin response, but may also modulate immune response against co-administered proteins, giving rise to mucosal (local) or systemic response, which indicates the influence of dietary lectins on immune function. Some lectins are also known for their immunomodulatory effects on peripheral blood lymphocytes (PBLs) and splenocyte/thymocytes by their ability to induce proliferation. The lectins selected for the present study have following specificities: oligo-(*N*-acetyl-D-glucosamine) specific potato lectin (*Solanum tuberosum* agglutinin; STA), *N*-acetyl-D-galactosamine specific horse gram lectin (*Dolichos biflorus* agglutinin; DBA), and mannose-specific garlic lectins (*Allium sativum* agglutinins-ASA I and ASA II). Since lectins are often the major proteins in many plant foods and are found to interact with the surface glycans of many cells of the immune system, it is interesting to study the modulatory aspects of certain dietary lectins in order to understand their importance and role in hypersensitivity and immunogenicity.

The research work carried out forms the subject matter of the thesis. The thesis is divided into seven chapters. Chapter 1 begins with a general account on lectins followed by an overview of the current literature on dietary lectins, hypersensitivity, modulatory effects in relation to hypersensitivity and immunogenicity. The chapter highlights the aims and scope of the present study. Chapter 2 deals with the effect of *Dolichos biflorus* agglutinin (DBA) which is an important dietary lectin in horse gram, on the degranulation of mast cells and basophils of atopic subjects. DBA induces activation of mast cells *in vivo* in a sub-population (21%) of atopic subjects. Two subjects have been identified as having food allergy to horse gram based on the presence of DBA-specific IgE with a positive correlation to basophil histamine release. This is the first study of food allergy to horse gram, and DBA has been identified as an allergen (designated Dol b Lectin). Chapter 3 describes the purification of potato lectin (*Solanum tuberosum* agglutinin; STA) an arabinose rich glycoprotein, utilizing the pentose

colorimetric assay for monitoring its presence. A simpler and shorter procedure has been developed for the purification of potato lectin from tubers. The hemagglutination inhibition assay using glycoproteins revealed its binding to glycoproteins. Binding of potato lectin to chitobiose core of N-linked glycoproteins has been confirmed by glycoprotein-binding assay using model glycoproteins. Chapter 4 deals with the study of potato lectin in its ability to release histamine from basophils *in vitro* and mast cells *in vivo* from non-atopic and atopic subjects. SPT on 110 atopic subjects using STA showed 39 subjects positive (35%); however, none showed STA-specific IgE; among 20 non-atopic subjects, none were positive by SPT. Maximal histamine release was found to be 65% in atopic subjects (n=7) as compared to 28% in non-atopic subjects (n=5); the release was specifically inhibited by oligomers of *N*-acetyl-D-glucosamine and the release correlates well with serum total IgE levels ($R^2$ = 0.923). It is speculated that higher intake of potato may increase the clinical symptoms as a result of non-allergic food hypersensitivity in atopic subjects.

Chapter 5 describes the purification of the mannose-specific garlic (*Allium sativum*) agglutinins (ASA I and ASA II), and their interaction with certain immunoresponder cells involved in hypersensitivity. ASA II exhibited one-third the specific hemagglutination activity of ASA I. Both ASA I and ASA II were found to induce histamine release from leukocytes of atopic and non-atopic subjects; ASA I (61%) is more potent compared to ASA II (38%). Heat-processed garlic extract was found to retain ~70% of the biological activity of raw garlic extract (RGE) as assessed by hemagglutination and glycoprotein binding assays. Chapter 6 examines the mitogenic potential of potato and garlic lectins towards human PBLs, murine splenocytes and thymocytes. ASA I, ASA II, and RGE showed 3.5, 1.5, and 2.5 fold increase, respectively, in proliferation index as compared to control untreated human PBLs. On the other hand, potato lectin, raw- and heat-processed potato extracts do not show any significant proliferative effect on both human PBLs, and murine splenocytes/thymocytes. Chapter 7 addresses the immunogenicity and adjuvant properties of garlic lectins. By intradermal and intranasal administration, ASA I show 4.2 and 3.5 fold increase, and ASA II shows 2.8 and 1.2 fold increase, respectively, in IgG response as compared to the reference protein ovalbumin (OVA) a poor antigen. ASA I showed a significant anti-OVA IgG response and was identified as a mucosal adjuvant against the co-administered antigen ovalbumin. The thesis ends with a comprehensive summary and conclusions emphasizing the salient aspects of the present study.

**List of publications from the present study**

1. **Pramod SN** and Venkatesh YP. Utility of pentose colorimetric assay for the purification of potato lectin, an arabinose-rich glycoprotein. *Glycoconjugate Journal* 2006; **23**: 481-488.
2. **Pramod SN**, Krishnakantha TP and Venkatesh YP. Effect of horse gram lectin (*Dolichos biflorus* agglutinin) on degranulation of mast cells and basophils of atopic subjects: Identification as an allergen. *International Immunopharmacology* 2006; **6**: 1714-1722.
3. **Pramod SN**, Venkatesh YP and Mahesh PA. Potato lectin activates basophils and mast cells of atopic subjects by its interaction with core chitobiose of cell-bound non-specific immunoglobulin E. *Clinical and Experimental Immunology* 2007; **148**: 391-401.
4. **Pramod SN**, Fatima Clement and Venkatesh YP. Modulatory effects of mannose-specific lectins from garlic (*Allium sativum*) on the immune cells involved in atopy (To be submitted to **FEBS journal**).
5. **Pramod SN,** Muthukumar SP and Venkatesh YP. Assessment of immunogenicity and adjuvanticity of garlic lectins by their mucosal and systemic responses (To be communicated).

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## ABBREVIATIONS

| | |
|---|---|
| AP | alkaline phosphatase |
| ASA | *Allium sativum* agglutinin |
| BCIP | 5-bromo-4-chloro-3-indolyl phosphate |
| BSA | bovine serum albumin |
| CBB | coomassie brilliant blue |
| cNOS | cytochrome nitric oxide synthase |
| Con A | concanavalin A |
| DBA | *Dolichos biflorus* agglutinin |
| DSA | *Datura stromanium* agglutinin |
| ELISA | enzyme-linked immunosorbant assay |
| FCO | ferric chloride-orcinol |
| FCS | fetal calf serum |
| Gal | galactose |
| GalNAc | N-acetyl-D-Galactosamine |
| Glc | glucose |
| GlcN | D-Glucosamine |
| GlcNAc | N-acetyl-D-Glucosamine |
| GPC | gel permeation chromatography |
| GPE | garlic powder extract |
| HA | hemagglutination |
| HDM | house dust mite |
| HR | histamine release |
| HEPES | *N*-(2-hydroxyethyl) piperazine-*N*'-(2-ethane sulfonic acid) |
| HPGE | heat processed garlic extract |
| RP-HPLC | reverse phase high performance liquid chromatography |
| HPPE | heat processed potato extract |
| HRP | horseradish peroxidase |
| Hyp | hydroxyproline |
| i.d. | intradermal |
| i.n. | intranasal |
| LEA | *Lycopersicon esculentum* agglutinin |
| LPS | lipopolysaccharides |

| | |
|---|---|
| Man | mannose |
| MTT | 3-(4,5-dimethylthiozol-2-yl) 2,5-diphenyl tetrazolium bromide |
| NAFH | non-allergic food hypersensitivity |
| NC | nitrocellulose |
| NBT | nitroblue tetrazolium |
| NKC | natural killer cell |
| NO | nitric oxide |
| OPD | *o*-phenylenediamine |
| OVA | ovalbumin |
| PAGE | polyacrylamide gel electrophoresis |
| PBLs | peripheral blood lymphocytes |
| PBS | phosphate buffered saline |
| PECs | peritoneal exudate cells |
| PHA | phytohemagglutinin |
| PSS | physiological salt solution |
| PWM | poke weed mitogen |
| RGE | raw garlic extract |
| ROS | reactive oxygen species |
| RPE | raw potato extract |
| S.A | sialic acid |
| SDS | sodium dodecyl sulfate |
| SPT | skin prick test |
| STA | *Solanum tuberosum* agglutinin |
| TCA | trichloroacetic acid |
| TBS | Tris buffered saline |
| Tris | Tris (hydroxymethyl) aminomethane |
| WGA | wheat germ agglutinin |
| w/v | weight by volume |
| v/v | volume by volume |

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# SYNOPSIS

**Synopsis of the thesis submitted to the University of Mysore for the award of PhD degree under the faculty of Biochemistry**

**Title of thesis: Studies on the modulatory aspects of certain dietary lectins in relation to hypersensitivity and immunogenicity**

**Candidate: S. N. Pramod**

Lectins are carbohydrate binding-proteins or glycoproteins of non-immune origin. Studies on the interaction of dietary lectins with cells of the immune system are rather limited in the literature. The interaction of lectins with basophils and mast cells resulting in release of histamine and other biological mediators has been studied with only a few lectins, and is, in many respects, similar to the interaction of food allergens with mast cells/basophils of allergic subjects. Adverse reactions caused by the activation of mast cells/basophils by lectins present in food sources generally fall under 'non-allergic food hypersensitivity' reactions, commonly known as false food allergy. Dietary lectins can either crosslink the carbohydrates on cell-bound IgE or directly the carbohydrates of cell surface glycoproteins/glycolipids including Fc epsilon receptors (FcεRI or RII) on basophils or mast cells. In both these cases, the end results are similar to the food allergen-specific IgE interaction seen in food allergy. Therefore it is important to address the role of plant dietary lectins in mediating non-allergic food hypersensitivity reactions.

Lectins may modulate the immune system in various ways. Dietary lectins can lead to the generation of anti-lectin immune response, but may also modulate immune response against co-administered proteins, the so called adjuvant effect. Moreover, lectins may also be able to polarize the immune response towards certain effector functions. In addition, dietary lectins may give rise to a mucosal (local) or a systemic response, which shows the influence of dietary lectins on immune function. Another way of categorizing immunomodulatory effects is the *in vitro* mitogenicity of lectins, which is typically a result of their ability to induce proliferation of lymphocytes in lymphoid organs

or blood. Lectins are commonly used to induce lymphocyte proliferation in experimental immunology.

The present study is focused on the modulatory effects of certain purified dietary lectins on the cells of the immune system (basophils, mast cells and lymphocytes), which will aid in delineating the role of lectins in non-immune mediated food hypersensitivity reactions (false food allergy), and their modulatory effects on immune cells to induce immunogenicity. Since lectins are often the major proteins in many plant foods and are found to interact with the surface glycans of many organ systems, it is interesting to study the modulatory aspects of certain dietary lectins to understand their importance and role in relation to hypersensitivity and immunogenicity.

The lectins selected for the present study have following specificities: oligo-(*N*-acetyl-D-glucosamine) specific potato lectin (*Solanum tuberosum* agglutinin; STA), *N*-acetyl-D-galactosamine specific horse gram lectin (*Dolichos biflorus* agglutinin; DBA) and mannose-specific garlic lectins (*Allium sativum* agglutinins - ASA I and ASA II). Potato lectin and garlic lectins were isolated in the laboratory by published procedures using slight modifications, whereas purified horse gram lectin was procured from a commercial source. The present study focuses on studying the modulatory aspects of these dietary lectins with the immune cells involved in hypersensitivity and immunogenicity under the following objectives:

(i) To investigate the effect of certain purified lectins on cells of the immune system involved in allergic reactions (specifically on basophils and mast cells) with a view to understand their role in non-immune mediated food allergic reactions (false food allergy).
(ii) To study the immunomodulatory effect of purified proteins (native and heat-processed potato lectin, and garlic agglutinins I, II) on human and murine lymphocytes.
(iii) To study the mucosal adjuvant effects of garlic lectins (*Allium sativum* agglutinins I and II) in order to assess the immunogenicity of these lectins.

The main focus of the present study is to provide valuable information on how dietary lectins with different carbohydrate specificities activate the effector immune functions of the cells of immune system, which are involved in hypersensitivity and immunogenicity reactions. The research work carried out towards achieving the objectives forms the subject matter of the thesis. The thesis is divided into seven chapters.

**Chapter 1: General Introduction**

The chapter begins with a general account on lectins followed by an overview of current literature on dietary lectins, hypersensitivity, and modulatory effects of dietary lectins in relation to hypersensitivity as well as immunogenicity. Chapter 1 highlights the aims and scope of the present study.

The subsequent chapters have a general format of Introduction followed by sections on Methodology, Results, Discussion and a brief Summary and conclusions. Literature cited has been listed in alphabetical order at the end (following Chapter 7) as Bibliography.

**Chapter 2: Effect of horse gram lectin (*Dolichos biflorus* agglutinin) on degranulation of mast cells and basophils of atopic subjects**

Horse gram (*Dolichos biflorus*) is widely consumed in the tropical south Asian countries including rural areas of India. Since *Dolichos biflorus* agglutinin (DBA) is an important dietary lectin in horse gram, a study has been made of its effect on the degranulation of mast cells and basophils of atopic subjects. Skin prick test (SPT) was performed with 100 µg/mL of DBA. DBA-specific IgE was detected by dot-blot, and ELISA. Histamine release (HR) assay was carried out using leukocytes from non-atopic and atopic subjects, and rat peritoneal exudate cells. Among the atopic group, 10 of 48 subjects (21%) were found to be positive by SPT to DBA, and none were positive in the non-atopic group (n=20). Two subjects out of the ten who tested positive for DBA by SPT were found to be sensitized to DBA as revealed by the presence of specific IgE by

ELISA and dot-blot. The HR was found to be 2-3 folds higher in DBA-allergic subjects than in non-atopic and atopic subjects. Basophil HR by DBA was found to be similar in both non-atopic and atopic subjects. However, DBA induces activation of mast cells *in vivo* in a sub-population (21%) of atopic subjects. Two subjects have been identified as having food allergy to horse gram based on the presence of DBA-specific IgE with a positive correlation to basophil HR. This is the first study of food allergy to horse gram, and DBA has been identified as an allergen, and designated Dol b Lectin.

**Chapter 3: Purification of potato lectin utilizing pentose colorimetric assay and development of a glycoprotein-binding assay**

Potato lectin (*Solanum tuberosum* agglutinin, STA) is an unusual glycoprotein containing approximately 50% carbohydrates by weight. Of the total carbohydrates, 92% is contributed by L-arabinose, which are *O*-linked to hydroxyproline residues. The ferric chloride-orcinol assay (Bial's test), which is specific for pentoses, has not been used for the detection of pentoses in bound form as it occurs in Solanaceae lectins (potato, tomato, and datura lectins). Utilizing the pentose colorimetric assay for monitoring the presence of potato lectin, a simpler and shorter procedure for the purification of this lectin from potato tubers has been developed. It involves only two chromatographic steps, namely, gel permeation on Sephadex G-75 and cation exchange on SP-Sepharose FF following ammonium sulfate precipitation of raw potato extract (RPE). The yield of potato lectin (1.73 mg per 100 g potato tuber) was twice compared to the yields reported in earlier procedures. Although potato lectin is well known for its specificity to free trimers and tetramers of *N*-acetyl-D-glucosamine (GlcNAc), it possesses a similar specificity to the core $(GlcNAc)_2$ of N-linked glycoproteins. The hemagglutination inhibition assay using glycoproteins revealed its binding to glycoproteins. Binding of potato lectin to chitobiose core of N-linked glycoproteins has been confirmed by glycoprotein-binding assay using horseradish peroxidase and avidin-alkaline phosphatase in ELLSA (enzyme-linked ligand sorbet assay) format. Heat-processed potato extract (HPPE) was observed to retain ~50 % of the biological activity

as compared to raw potato extract, but the isolation of lectin from HPPE proved to be unsuccessful. The utility of pentose assay appears to be a simple and convenient colorimetric assay for detecting pentose-rich glycoproteins in plant extracts and can be a useful tool to monitor their purification, as has been demonstrated for potato lectin in this study.

**Chapter 4: Modulatory effects of potato lectin (*Solanum tuberosum* agglutinin) on basophils and mast cells of atopic subjects**

A major factor in non-allergic food hypersensitivity could be the interaction of dietary lectins with mast cells and basophils. Since IgE contains 10-12% carbohydrates, lectins can activate and degranulate these cells by cross-linking the glycans of cell-bound IgE. The present chapter focuses on the effect of potato lectin (*Solanum tuberosum* agglutinin; STA) for its ability to release histamine from basophils *in vitro* and mast cells *in vivo* from non-atopic and atopic subjects. In this study, subjects were selected randomly based on case history and skin prick test (SPT) responses with pollen, and house dust mite extracts. SPT was performed with STA at 100 μg/mL concentration. Histamine release was performed using leukocytes from non-atopic and atopic subjects, and rat peritoneal exudate cells. SPT on 110 atopic subjects using STA showed 39 subjects positive (35%); however, none showed STA-specific IgE and among 20 non-atopic subjects, none were positive by SPT. Maximal histamine release was found to be 65% in atopic subjects (n=7) as compared to 28% in non-atopic subjects (n=5); the release was specifically inhibited by oligomers of *N*-acetyl-D-glucosamine and the release correlates well with serum total IgE levels ($R^2$ = 0.923). Binding of STA to *N*-linked glycoproteins (horseradish peroxidase, avidin, and IgG) was positive by dot blot and binding assay. Since potato lectin activates and degranulates both mast cells and basophils by interacting with the chitobiose core of IgE glycans, it is speculated that higher intake of potato may increase the clinical symptoms as a result of non-allergic food hypersensitivity in atopic subjects.

**Chapter 5: Modulatory effects of mannose-specific lectins from garlic (*Allium sativum*) on the immune cells involved in atopy**

The major proteins in garlic bulbs are alliinase and the low mol. wt. mannose-specific agglutinins or lectins ($ASA_{25}$). $ASA_{25}$ represents two agglutinins devoid of glycans: ASA I (a heterodimer), and ASA II (a homodimer). Although a vast amount of information is available on the structure and carbohydrate-specificity of garlic agglutinins, information on the effects of garlic agglutinins on the cells of the immune system is rather scarce. This study describes the purification of the mannose-specific *Allium sativum* agglutinins (ASA I and ASA II), and their interaction with certain immunoresponder cells involved in hypersensitivity. The agglutinins were purified from raw garlic extract (RGE) using a combination of gel filtration (Sephadex G-50) and cation exchange chromatography. Commercial garlic powder extract showed a similar specific hemagglutination activity and glycoprotein binding, as compared to RGE. Purified ASA I and II showed higher binding for the glycoproteins (HRP and avidin-alkaline phosphatase) compared to the raw extract, and their binding ability is comparable to that of the prototype mannose/glucose-specific lectin, concanavalin A (Con A). ASA II exhibited one-third the specific hemagglutination activity of ASA I. These dietary lectins from garlic have been chosen for studying their effects on human leukocytes *in vitro* and mast cells *in vivo*. Both ASA I and ASA II were found to induce histamine release (HR) from leukocytes of atopic and non-atopic subjects; maximal release was seen at 1 $\mu$g/mL concentration. ASA I is more potent compared to ASA II, and HR by ASA I in the case of atopic subjects (61%) is significantly higher compared to that of non-atopic subjects (36%). Heat-processed garlic extract was found to retain ~70% of biological activity compared to RGE as assessed by hemagglutination and glycoprotein binding assays.

**Chapter 6: Modulatory effects of potato and garlic lectins in relation to mitogenicity**

This chapter describes the modulatory effects of the mannose-specific garlic lectins (ASA I and ASA II) and oligo-GlcNAc specific potato lectin on lymphocytes in

relation to mitogenicity. Both the garlic lectins were found to be mitogenic towards human peripheral blood lymphocytes (PBLs) and murine splenocytes as well as thymocytes. ASA I, ASA II, and RGE showed 3.5, 1.5, and 2.25 fold increase, respectively, in proliferation index as compared to control cells (human PBLs), as determined by MTT assay. ASA I and II displayed 4.5 and 3 fold increase, respectively, in the proliferation activity with murine splenocytes and thymocytes compared to the respective control cells. The proliferation effects shown by ASA I (70-75%) and ASA II (45-50%) are comparable to those of the reference mitogens Con A (100%) and phytohemagglutinin (PHA) (85%). On the other hand, potato lectin, raw- and heat-processed potato extracts did not show any significant proliferation effect on both human PBLs, and murine splenocytes & thymocytes (<10% compared to Con A). ASA I and ASA II were unable to induce reactive oxygen species (ROS) from macrophages present in rat peritoneal exudate cells. However, RGE shows a significant increase in ROS generation indicating that this effect is not due to garlic lectins; heat-processed garlic extract lacks ROS generation property. Both potato and garlic lectins failed to induce the production of nitric oxide ($NO_2^-$) from human PBLs. From these observations, it is evident that garlic lectins possess mitogenicity for lymphocytes whereas potato lectin is devoid of the same.

**Chapter 7: Assessment of immunogenicity and adjuvanticity of garlic lectins by their mucosal and systemic responses**

Garlic lectins ASA I and II were tested for their ability to induce IgG response in BALB/c mice by intradermal and intranasal routes of administration. By intradermal administration, ASA I shows 4.2 fold increase, and ASA II shows 2.8 fold increase in IgG response as compared to the reference protein ovalbumin (a poor antigen). Both lectins were found to be potent immunogens that indicates the systemic measure of immune response. By intranasal route, only ASA I (3.5 fold) show a significant increase in IgG response than ASA II (1.2 fold) compared to the reference protein ovalbumin, which indicates the mucosal immune response. The anti-lectin IgG response for ASA I and

ASA II is considerably significant as compared to the reference protein ovalbumin. During the experimental duration, there was no reduction in the body weight of animals, suggesting that garlic lectins are not growth-retardant. Based on the spleen and thymus weights, and their indices, there was a slight increase in the reference ovalbumin group and significant increases were seen in the lectin groups, indicating the activation of lymphoid organs, by these dietary lectins. Anti-lectin antibody response by both intradermal and intranasal routes of administration increased after each booster dose, and the titer was found to be very significant in the lectins group than in the reference ovalbumin group. These observations indicate that dietary garlic lectins are potent immunogens. The mucosal adjuvant potential of ASA I and II was investigated for a poor antigen ovalbumin, and only ASA I was found to have a marked adjuvant activity whereas ASA II does not.

**General summary and conclusions**

The thesis ends with a comprehensive summary and conclusions stating highlights and the important observations made from the present study described in chapters 2 to 7.

# Chapter I

# General Introduction

## 1.1. Introduction

For over 5000 years, many plants have acquired a worldwide reputation in folklore as a formidable prophylactic and therapeutic medicinal agents (Wagner, 1990; 1999). Thousands of publications in the last century have confirmed the efficacy of plant components in the prevention and treatment of a variety of diseases, acknowledging and validating the traditional uses (Essman, 1984). Many favorable biological and pharmacological effects associated with the consumption and administration of plant components have been reported experimentally and clinically (Wagner and Proksch, 1985). In addition to the pharmacological activities, some plant extracts have been shown to be potential biological response modifiers (Hirao et al., 1987). Since certain diseases can be caused by immune dysfunction, modification of immune function by plant components contributes to the treatment and prevention of some diseases. Thus, some pharmacological effects of plant components might be mediated through immune modifications, which are commonly referred to as immunomodulatory functions (Klendler, 1987; Ip et al., 1994).

From a data bank obtained in 1997 (NAPRALERT and Phytodoc), over 200 citations on plant extracts or fractions and more than 30 citations on isolated products, describing immunomodulatory potential have been obtained. Of 130 plant crude drugs investigated, 90% belong to the higher plants and 10% to the class of mushrooms, fungi, algae and lichens (Brevoort 1996; Wagner, 1999). From more than 70% of the plants, ethanol and water extracts (decoction) or the powdered drugs have been investigated (Amagase, 2001). From the remaining 30%, more or less chemically defined fractions, i.e. polysaccharides (Akiyama et al., 1981), glycoproteins (Beuth, 1991), saponins (Lacaille-Dubois and Wagner, 1996), lignans, flavonoids or alkaloid-enriched fractions have been studied for their immunomodulatory potential (Eisemann et al., 1995) (Table 1.1). The parameters measured vary so widely that no conclusions could be drawn or comparisons could be made between the immunological potential of various plants. Different active components from the plants possess immunomodulatory activity both in vitro and in vivo. However, mechanisms of their actions are not sufficiently elucidated.

**Table 1.1. Classes of plant compounds with claimed immunomodulatory activity**

| Low molecular weight compounds | High molecular weight compounds |
|---|---|
| Alkyl amides | Proteins (lectins) |
| Phenolic compounds | Immunomodulatory proteins |
| Alkaloids | Peptides |
| Quinones | Polysaccharides |
| Saponins | Glycolipids |
| Sequiterpenes, di-and triterpenoids | |

The human body is continuously exposed to a series of stress factors, which more or less weaken the function of the immune system. The immunosuppression can be generated by severe bacterial and viral infections, cancer, environmental agents such as pesticides or allergens, malnutrition, psychic stress, or endogenic autoimmune reactions. Non-specific immunmodulation (Seiler, 1985) might be useful and very effective when the immune system of the host is impaired. Herbal medicines with immunostimulatory potential are appropriate for the prophylaxis and therapy of moderate infections (Essman, 1984). The oral applications of plant extracts are the appropriate form; for parenteral use, only indicated pure components are ideal drugs.

Immunostimulants or immunopotentiators are components leading predominantly to a non-specific stimulation of immunological defense mechanism. Most of them are not real antigens but antigenomimetics or so-called mitogens (Wagner, 1990). Non-specific and non-antigen dependent stimulants do not affect immunological memory cells and, since their pharmacological efficacy fades comparatively quickly, they have to be administered either at intervals or continuously. Some immunostimulants may also stimulate T-suppressor cells and thereby reduce immune resistance; hence the term immunomodulation or immunoregulation (Seiler, 1985), denoting any effect as a change of immune responsiveness, often used for describing the effect of plant extract on immune function.

Several dietary components (Table 1.1) are known to affect various functions of the immune system and to interfere with immune regulatory circuits (Strobel and Mowat,

1998). The immunobiological activity of carbohydrate-binding proteins of vegetable origin, the plant lectins, has long been recognized. Some plant lectins have been shown to be able to modulate important immune mechanisms (Heegaard and Muller, 1988), such as inflammatory reactions and effector functions (Toribio et al., 1985; Tchernychev and Wilchek, 1996).

### 1.2. Lectins

Lectins are proteins or glycoproteins of non-immune origin, which bind specifically to the glycan part of glycoconjugates (e.g., glycoproteins, glycolipids), oligosaccharides and polysaccharides in a sugar-specific manner (Sharon and Lis, 2004). They are a diverse group of multivalent sugar-binding proteins and often have two or more binding sites per molecule (Rini, 1995). They are abundant and are found to be ubiquitous in all forms of living matter, including bacteria and viruses (Wu et al., 1988). Lectins have been defined by their ability to bind specifically to carbohydrate and by their characteristic property of agglutinating different cell types viz., erythrocytes, leukocytes, tumor cells and bacteria (Goldstein et al., 1980; Green and Baenziger, 1987). The lectin-stimulated agglutination and effector functions suggest the possible involvement of the membrane glycoproteins or glycolipids containing specific carbohydrate residues in the interactions with the lectin (Wu et al, 1988).

An updated definition has lately been proposed; plant lectins have been redefined as “plant proteins possessing at least one non-catalytic domain, which binds reversibly to a specific mono- or oligosaccharide” (Peumans and Van Damme, 1995; 1998). A redefinition was necessary because monovalent lectins have been identified that cannot precipitate glycoconjugates or agglutinate cells (Peumans and Van Damme, 1998). By binding to specific carbohydrates on cell surfaces, lectins can elicit multiple changes in cell and tissue metabolic processes. Lectins bind specifically to different terminal carbohydrates and thus elicit various effects on the cells.

### 1.2.1. Plant lectins

Many plant species, including several important crop and food plants, contain carbohydrate-binding proteins better known as ‘lectins’, ‘agglutinins’, or ‘hemagglutinins’ (Van Damme et al., 1998b). By virtue of their specificity toward well-defined glycans, especially of animal origin, plant lectins are used intensively in glycoconjugate research as sensitive molecular probes.

In addition, many plant lectins are widely exploited in biological and biomedical research because of their unique biological activities on various animal and human cells (Sharon and Lis, 1972; 2004). Plant lectins were primarily regarded as bioactive proteins by Stillmark in 1888. As a consequence, the majority of all currently known lectins have been tested extensively to reveal their possible effects on cells and organelles. Until the discovery of the carbohydrate-binding activity of lectins, the underlying mechanism(s) of the biological activities of plant lectins remained enigmatic.

As soon as it became evident that the activity of lectins is based on the recognition and binding of specific sugar residues, the concept of lectin-receptor was introduced to explain the various activities of plant lectins in molecular terms (Sharon, 1993; Pusztai and Bardocz, 1996). Along with the discovery of many novel lectins, evidence accumulated that different plant lectins exhibit obvious differences with respect to their biological activities toward animal and human cells (Van Damme et al., 1998b). The differences were intimately linked to the structure of cell surface glycan receptor(s) of the respective lectins.

### 1.2.2. Occurrence of plant lectins

Lectins are usually considered a very extended group of plant proteins, because at first sight, the list of lectin-containing species is almost endless (Van Damme et al., 1998b). Plant lectins are not typical seed proteins. They have been found in virtually all types of vegetative tissues, and according to the recent compilations, the number of documented non-seed lectins surpasses that of the seed lectins (Rudiger and Gabius, 2001; Van Damme et al., 1998a). It is also worth noting that several of the most

intensively used lectins are purified from vegetative tissues (e.g., the bulb lectin from snowdrop and the bark lectin from elderberry).

The concentration of lectins in seeds and vegetative tissues varies strongly. Seed lectins usually account for 1 to 10% of the total seed protein (Liener, 1997). In some species, even higher values have been reported (up to 50%), whereas in others the lectins are barely detectable with the techniques currently used. The same holds true for the lectin concentration in vegetative tissues. Many plants accumulate large quantities of lectins in their vegetative storage tissues. Lectins usually account for 1 to 20% of total protein content of bark, bulbs, tubers, rhizomes and corns (Rudiger and Gabius, 2001). In some cases values up to 50% have been reported. Fruits, leaves, flowers, ovaries, roots, and other tissues usually contain low levels (<1% of the total protein) of lectin(s). It should be mentioned that the lectin content of both seeds and vegetative tissues is often developmentally regulated (Peumans and Van Damme, 1995).

**1.2.3. Classification of plant lectins**

The definition of lectins includes a wide range of proteins; plant lectins are divided into five major families of structurally or functionally related proteins: legume lectins, monocot mannose-binding lectins, jacalin-related lectins, chitin-binding lectins and type-4 ribosome–inactivating proteins (Table 1.2). In addition to these five families, three other families are now also recognized (Van Damme et al., 1998a). Another way of classifying lectins is based on their structure, and they fall into three classes: merolectins, hololectins, and chimerolectins (Sharon and Lis, 2003; 2004). Merolectins consist of only one carbohydrate-binding domain and hence, are incapable of agglutinating cells. Hololectins, the class in which the majority of plant lectins belong have at least two domains, composed of only carbohydrate-binding domains as opposed to chimerolectins. The latter consist of one or more carbohydrate-binding domains and, in addition, an unrelated domain with well-defined biological activity such as enzymatic activity.

**Table 1.2. Occurrence, molecular structure and specificity of plant lectin families**

| Lectin Family | Occurrence | | Molecular structure | | Nominal specificity |
|---|---|---|---|---|---|
| | Taxonomic distribution | Approximate no. identified lectins | Protomer size (kDa) | Number of protomers | |
| Chitin-binding lectins | Ubiquitous Diverse | >50 | 30 | 1 or 2 | $(GlcNAc)_n$ |
| | Solanaceae | <10 | 50 | 2 | $(GlcNAc)_n$ |
| Jacalin related lectins | Monocots and dicots | <10 | 16 | 2 or 4 | Gal/GalNAc Mannose/Maltose |
| Legume lectins | Legumes | >100 | [(30-X) + X] | 2 or 4 | Mannose/Glucose Fucose, $(GlcNAc)_n$ Gal/GalNAc Sialic acid, |
| Monocot mannose-binding lectins | Liliales, Arales Orachidales Bromeliales | >100 | 12 or 30 or [(30-X) + X] | 1,2,3 or 4 | Complex mannose |
| Type 4 RIP | Monocots and dicots | >20 | [30-s-s-35] | 1,2 or 4 | Gal/GalNAc or Sia$\alpha$2-6Gal/GalNAc |

Gal: Galactose, GalNAc: N-acetyl-D-galactosamine, $(GlcNAc)_n$: polymer of N-acetyl-D-glucosamine (Peumans and Van Damme, 1998; Pusztai and Bardocz, 1996); RIP: ribosome-inactivating protein (Peumans and Van Damme, 1998).

### 1.2.4. Structure of plant lectins

Despite the obvious three-dimensional diversity observed in the different families of plant lectins, some common structural requirements are apparently necessary for specific sugar-binding activity. It is noteworthy that $\beta$-sheets, irrespective of their overall three-dimensional organization, occur predominantly, if not exclusively, in all proteins characterized as lectins. The overall folding of the monomers, which are typically built from $\beta$-sheets connected by turns and loops, creates very tight structural scaffolds.

Carbohydrate-binding sites emerge from these scaffolds as depressions or pockets resulting from the convergence of flexible loops (Edelman et al., 1972; Sharon and Lis 2004). In addition, the flattened shape of the monomers favors their subsequent oligomeric arrangement, which, in turn, confers a multivalent character to most lectins (Weis and Drickamer, 1994; Drickamer 1988). This quaternary arrangement strongly enhances the ability of lectins to bind to sugars and especially complex N-linked sugars, and is therefore very important for plant proteins involved in recognition processes (Van Damme et al., 1998b).

### 1.2.5. Lectins in the human diet (Dietary lectins)

Plant and animal materials used as foodstuffs contain lectins, some of which are denatured by cooking. There are, however, still active lectins in the diet as when foodstuffs are eaten raw (uncooked) and some lectins are still active after cooking or processing (Peumans and Van Damme, 1998). In general, most lectins are inactivated by heat treatment such as those involved in commercial processing or household cooking. This, however, still leaves foodstuffs like juices, tomatoes, raspberries, garden peas, salad ingredients, spices, dry cereals, and roasted nuts in which lectins are consumed in an agglutinative active form. Nachbar and Oppenheim (1980) found, by survey of the literature in combination with their own work, that 82 different edible plants contained agglutinative-active lectin.

As a generalization, lectins appear to be refractory to hydrolysis by digestive enzymes. There is evidence that lectins, as phylogenetically distinct as those from wheat (Brady et al., 1978) and tomato fruit (Kilpatrick et al., 1985), can travel from the human mouth to the colon with a substantial proportion of the molecule remaining in intact form. Similar resistance to the digestive process has been found for various lectins introduced into the stomach of rodents (Pusztai, 1991). It seems likely, therefore, that dietary lectins may be biologically active throughout the human alimentary tract and any activities they may possess therein could be modified by inhibitory sugars in the diet (Wang et al., 1998). Lectins may also be neutralized by provoking an IgA response;

certainly salivary IgA could interact with dietary peanut agglutinin, pea lectin and wheat germ agglutinin (Gibbsons and Dankers, 1983; 1986). Although lectins have been isolated and well characterized from other edible legumes, their toxicity upon oral administration, hence their nutritional significance remains unknown. The human diet emphasizing the degree to which the human population is exposed to dietary sources of lectins is accountable. However, the absence of any study involving the oral intake of lectins purified from these sources, and their possible physiological significance is still unclear.

### 1.2.6. Interactions and survival of dietary lectins in the digestive tract

As part of the normal turnover of the gut epithelium, cells are shed from the villus tips into the lumen and most cellular material is then digested and recycled. The presence of lectins attached to these cells does not interfere with the breakdown of cell contents, but the liberated lectins can move further down the gut and bind to the next receptor with an appropriate carbohydrate moiety (Pusztai, 1991; 1993). Although lectin binding is most frequently studied in the small intestine, similar binding can occur throughout the entire digestive tract, from the stomach to the distal colon (Pusztai et al., 1990). However, as surface glycosylation varies in the different functional parts of the gut, lectin binding is not uniform in the digestive tract.

#### 1.2.6.1. Mouth and throat

Dietary lectins interact with buccal epithelial cells and/or bacteria or viruses. Certainly for two lectins occurring in normal diets (peanut agglutinin and wheat germ agglutinin), binding to gut-epithelial cells has been noted (Gibbson and Dankers, 1983). These lectins also bind to oral bacteria and it is evident that they have the potential to alter oral bacterial ecology and may be of relevance to periodontal diseases. The influence of sugars on lectins in the diet could influence the nutritional significance of these lectins in the gut. Dietary lectins, in general, bind surface glycans of brush-border epithelial cells causing damage to the villi, which includes disarrangement of the

cytoskeleton, increased endocytosis, and shortening of the microvilli (Liener, 1986; Sjolander et al., 1986; Pusztai, 1993) (Fig 1.1). The structural changes induced by dietary lectins on gut and intestinal epithelial cells elicit functional changes including increased permeability (Sjolander et al., 1984), which may facilitate the passage of undegraded dietary lectins into systemic circulation (Pusztai, 1993).

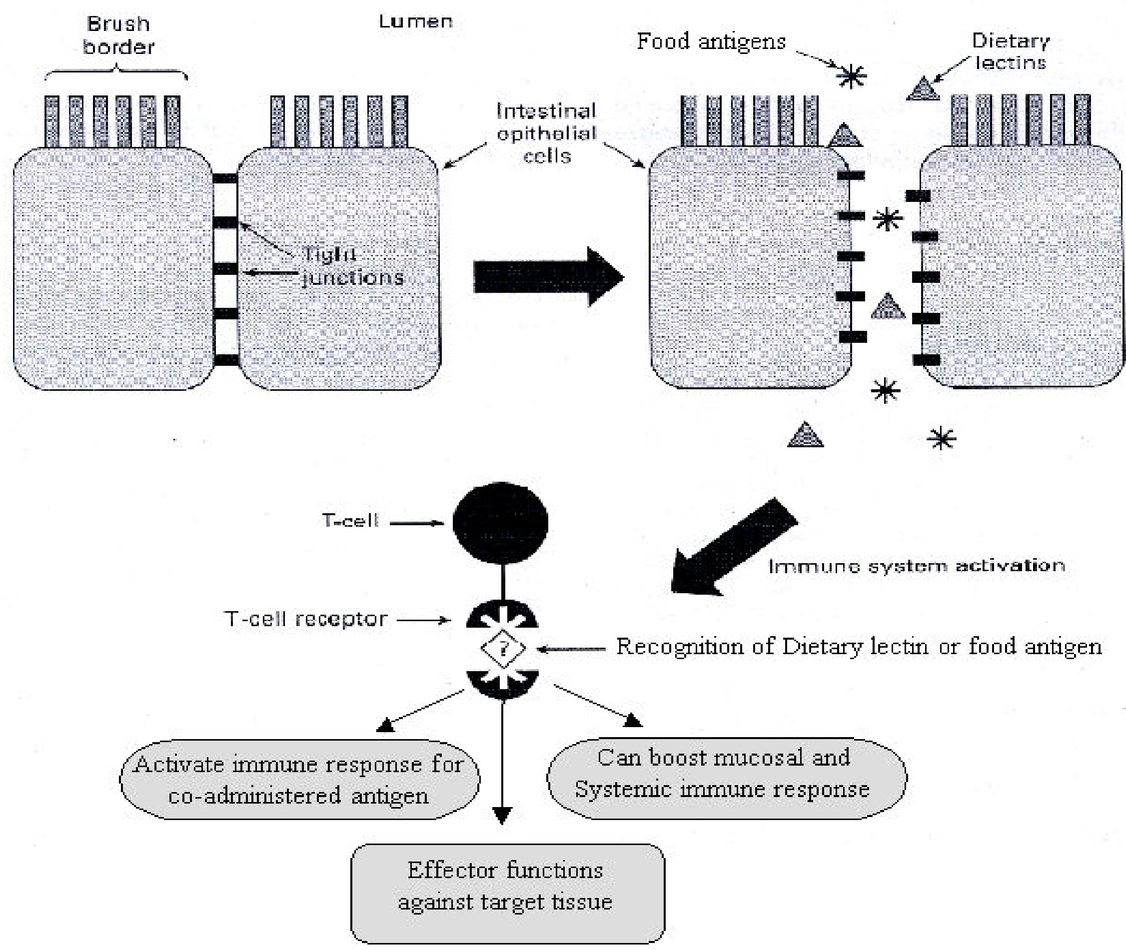



*Fig. 1.1. A diagrammatic illustration of how dietary lectins may hypothetically interact with the gut and immune system. Dietary lectins may (1) increase the gut permeability allowing increased passage for itself and co-administrated food antigens to the periphery, (2) stimulate T-cell proliferation, and (3) stimulate the production of inflammatory cytokines (Cordain et al., 2000).*

#### 1.2.6.2. Stomach and intestines

The interaction of numerous lectins, particularly PHA, with the rat small intestine has been extensively studied (Pusztai, 1991) (Table 1.3). These studies led to the following generalization: (a) to a significant extent, lectins reach the gut in a structurally intact and biologically active form, (b) lectins may bind to the gut wall and act as local growth promoters, but also as systemic toxins, and (c) the toxic effects are not observed in germ-free animals, suggesting bacterial overgrowth and toxin production is crucial (Brady et al., 1978). In rats, dietary wheat germ agglutinin is rapidly transported across the intestinal wall into the systemic circulation where it is deposited in blood and lymphatic vessel walls (Pusztai, 1993).

Table.1.3. Survival and binding of pure lectins to the small intestinal mucosa

| Lectin | Specificity | Binding | Recovery (%) | Toxicity | |
|---|---|---|---|---|---|
| | | | | Peritonial* | Oral# |
| PHA (*Phaseolus vulgaris*) | Complex | +++ | > 90 | + | + |
| Con A (*Canavalia ensiformis*) | Man/Glc | + | > 90 | + | + |
| GNA (*Galanthus nivalis*) | Man | - | > 90 | + | – |
| SNA-II (*Sambucus nigra*) | GalNAc | +++ | > 60 | + | – |
| SBA (*Glycine max*) | GalNAc/Gal | ++ | > 60 | + | – |
| LEL (*Lycopersicon esculentum*) | $(GlcNAc)_3$ | + | 40-50 | ? | ? |
| WGA (*Triticum vulgare*) | GlcNAc | ++ | 50-60 | + | + |
| PSL (*Pisum sativum*) | Man/Glc | + | 30-40 | ? | ? |
| VFL (*Vicia faba*) | Man/Glc | ± | 20-30 | ? | ? |
| DGL (*Dioclea grandiflora*) | Man/Glc | ± | 18-20 | ? | ? |

The table describes the amounts of lectin surviving in the stomach and small intestine which were estimated from luminal washings and supernatants of the tissue homogenized with 0.1 M solution of the appropriate carbohydrates in PBS, pH 7.6 when rats were intragastrically intubated with 10 mg of individual lectins.
*Death resulted from the intraperitoneal injection of crude extract or purified lectin.
#Growth inhibition caused by adding purified lectin to the diet of experimental animals.
(Taken from Pusztai and Bardocz, 1996; Cordain et al., 2000).

### 1.2.7. Toxicity and biological effects of lectins in foods

Lectins bind with high affinity to oligosaccharides, which are absent in plants, but abundant in bacteria as polysaccharides of bacterial cell walls and in animals as constituents of glycoproteins and glycolipids in cell membranes (Peumans and Van Damme, 1996; Van Damme et al., 1998a). Many of the membrane-integrated glycoproteins function as receptors for hormones and cytokines or are involved in cell-cell recognition (Muraille et al., 1999). Lectins may therefore mimic a natural ligand of a receptor or inhibit binding of a natural ligand and thereby evoke a variety of systemic and local effects (Tchernychev and Wilchek, 1996), such as cell division and growth, cell maturation and cell death (Ohba et al., 2003). Lectins are often detected by their ability to agglutinate red blood cells; this biological activity is, however, not central with respect to most lectins found in human food and animal feed.

A very important *in vivo* biological activity is the striking effects that some dietary lectins have on gut function. Such lectins react with the surface epithelium of the digestive tract, and are in some cases mitogenic for enterocytes (Banwell et al., 1993; Otte et al., 2001). Phytohemagglutinin (PHA) is a powerful growth factor for the gut cells and by interacting with the brush border epithelial receptors induce extensive proliferation of epithelial cells (Banwell et al., 1993). It has been shown that PHA reversibly induces hyperplasic and hypertrophic growth of the small bowel (Bardocz, 1996). In addition to its role as a growth factor for the gut, PHA induces enlargement of the pancreas (Pusztai et al., 1995). Many lectins are very potent exogenous growth signals; some can even mimic the action of major metabolic hormones and growth factors (Pusztai, 1993). The effects of some lectins on the gut and other parts of the body are especially important because lectins account for a relatively large fraction of plant proteins.

#### 1.2.7.1. Interference with the absorption of nutrients

The major consequences of lectin damage to the intestinal mucosa appear to be a serious impairment in the absorption of nutrients across the intestinal wall (Frokiaer et

al., 1997). This was first demonstrated *in vitro* with isolated intestinal loops taken from animals that had been fed raw black beans, or a lectin purified therefrom displayed a significant decrease in the rate at which glucose was transported across the intestinal wall. It would appear that the effect of lectins on the absorptive ability of the intestine is most likely a consequence of changes in the intestinal permeability (Sjolander et al., 1984; Greer and Pusztai, 1985). These findings may have important implications with reference to allergic reactions to foods containing lectins. Lectins can also affect brush border hydrolyses, which play a vital role in the digestion of proteins and carbohydrates (Triadou and Audran, 1983; Erickson and Kim, 1983). The interaction of lectins with brush border membranes resulted in inhibition of brush border peptidases (Kim et al., 1976) and enterokinase (Rouanet et al., 1983) *in vitro*.

#### 1.2.7.2. Bacterial colonization

The precise mechanism whereby lectins induce the colonization of the small intestine is not known. It has been suggested that lectins can cause an impairment of the immunological suppression of the growth of certain bacteria (Wilson et al., 1980). Lectins are responsible for inducing aggregation of bacterial cells and help their growth due to malabsorption of nutrients. Lectins because of their polyvalency, bind to receptor sites on the brush border as well as bacteria, and thus serve to “glue” bacteria to the intestines (Banwell et al., 1983; 1993).

#### 1.2.7.3. Internalization of lectins

Lectin-induced damage to the intestinal mucosa alters their permeability so that normally innocuous intestinal bacteria, or the endotoxins that they produce, gain entrance into the bloodstream and produce toxic systemic effects (Pusztai et al., 1989). It was also considered the possibility that lectins, either intact or partially digested may themselves enter the circulatory system to exert toxic reactions such as inhibition of protein synthesis, local or systemic immune hypersensitivity (Mitchell and Clarke, 1979),

or tissue damage. The immunochemical demonstration of the presence of lectins in blood has provided support to this theory (Wang et al., 1998).

### 1.2.8. Systemic effect of dietary lectins

Lectins may influence systemic effects by two different but possibly simultaneous mechanisms. Lectins can indirectly influence the endocrine system of the body by binding to the neuroendocrine cells of the gut and stimulating the secretion of gut peptide hormones into systematic circulation (Liener, 1986; Pusztai, 1993). Alternatively, lectins can be transmitted through the gut wall into the blood circulation and thus may directly influence peripheral tissues and body metabolism by mimicking the effects of endocrine hormones. The organs most often affected are the pancreas, skeletal muscle, liver, kidneys, spleen and thymus. Dietary lectins influence the structure and function of both enterocytes and lymphocytes (Liener, 1986; Pusztai, 1993), as they have potent anti-nutritional properties, and most of them are heat stable and resistant to digestive proteolytic breakdown in both rats and human subjects (Brady et al., 1978) and have been recovered intact and biologically active in human feces (Brady et al., 1978).

Lectins, in general, bind surface glycans on gut and intestinal brush-border epithelial cells causing damage to the villi, which elicit functional changes, including increased permeability (Sjolander et al., 1984; Greer et al., 1985) which facilitate the passage of undegraded dietary lectins into systemic circulation (Pusztai, 1993). When the luminal concentration of intact dietary proteins is low, absorbed proteins generally elicit a minimal allergic response because of the limiting influence of T-suppressor cells. Due to their resistance to digestive proteolytic breakdown, the luminal concentrations of dietary lectins can be quite high; consequently their transport through the gut wall can exceed that of other dietary antigens by several orders of magnitude (Pusztai 1989), and absorbed dietary lectins can be presented by macrophages to competent lymphocytes of the immune system (Hruby et al., 1985; Ohba et al., 2003). Since dietary lectin escape proteolytic digestion and can cause increased gut and intestinal permeability, they are

able to cross the gastrointestinal barrier rapidly and enter the systemic circulation intact and are able to induce local, systemic, and pathophysiological effects on immune cells.

### 1.3. Dietary lectins and hypersensitivity or allergic reactions

The immune system serves to protect the host from external dangers; yet, inappropriate responses of this system can lead to disease. Common among these dysfunctions of the immune system are allergies and autoimmune diseases. It has often been suggested that dietary lectins might be involved in initiating these inappropriate immune responses (Strober and Fuss, 1999). Lectins mimic *in vivo* basophil or mast cell activation as that of allergen and may also influence allergy induction against another co-ingested protein. However, there have been only a few investigations on the influence of lectins on allergy induction and no general conclusions have been drawn from these reports, due to the use of different animal models and different lectins. Con A, LCA, PSA, RCA-I and PHA-E have been shown to be able to bind with high affinity to IgE from allergic patients (Shibasaki et al., 1992). These five lectins also induced release of histamine when leukocytes from allergic subjects were stimulated. WGA bound to IgE with low affinity, but still induced release of histamine (Shibasaki et al., 1992). As dietary lectins are able to bind to human IgE through its glycans, they may induce symptoms like those seen in allergy by cross-linking IgE molecules on mast cells or basophils.

#### 1.3.1. Hypersensitivity or inflammatory reactions

Although the immune system generally is protective, the same immunologic mechanisms that defend the host at times may result in severe damage to tissue. These damaging immunologic reactions are called as hypersensitivity reactions. Coombs and Gell (1975), classified hypersensitivity reactions into classified into four major types based on differences in the immune response. Although, current understanding of allergic diseases has grown vastly since then, this classification system remains useful even today. The 4 types are; immediate hypersensitivity (type I) reactions, cytotoxic

(type II) reactions, immune-complex mediated (type III) reactions and T cell-mediated or delayed (type IV) hypersensitivity. Immediate hypersensitivity reaction is the major out of the four types and is the basis of acute allergic reactions, which is responsible for triggering the allergic symptoms.

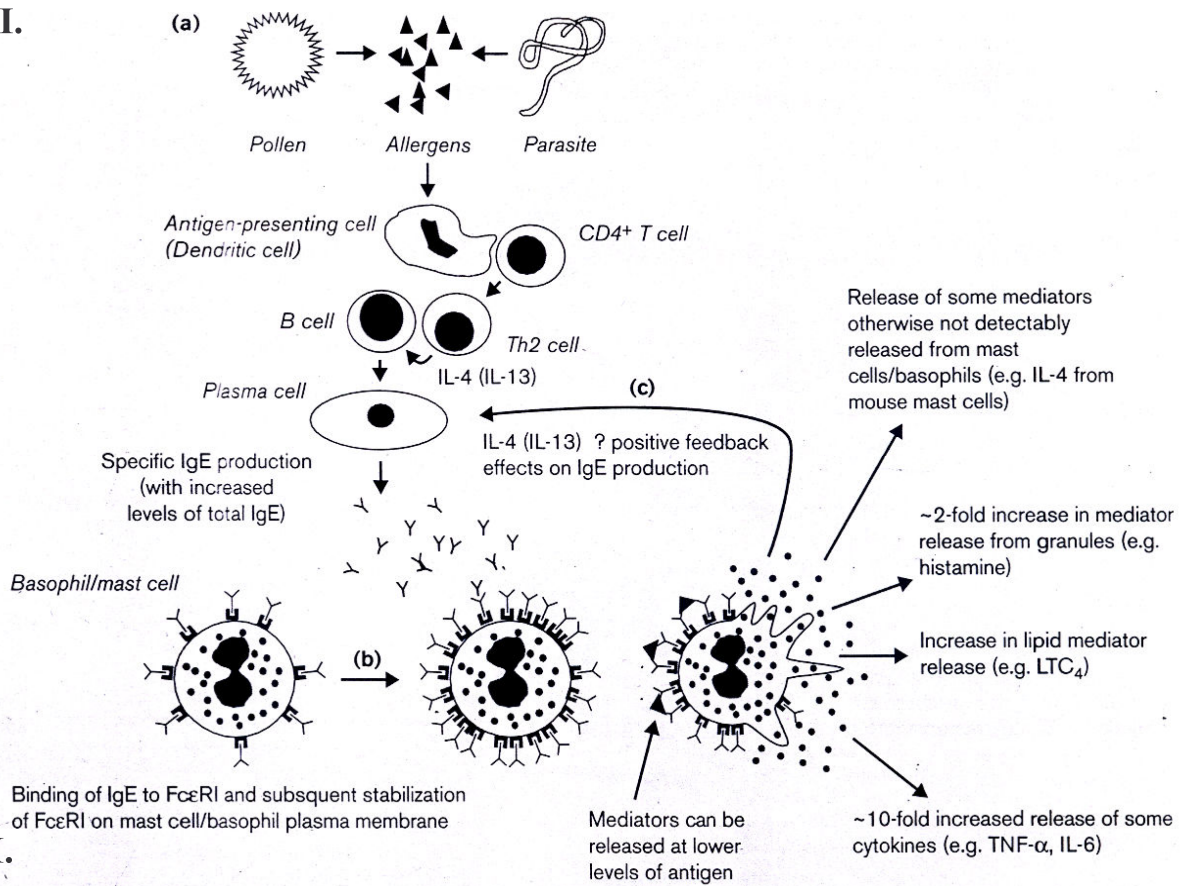
### 1.3.2. Immediate type IgE-mediated hypersensitivity

IgE-mediated inflammation occurs when an antigen (allergen) binds to the IgE antibodies that occupy the FcεRI receptor on mast cells or basophils. Within minutes, the binding complex of allergen, IgE, and FcεRI causes mast cell or basophil degranulation, which triggers a non-cytotoxic, energy-dependent release of certain preformed mediators (Helm and Burks, 2000; Kay, 2001). Subsequently, the degranulated cell begins to synthesize and release additional mediators *de novo*. The result is a two-phase response; an initial immediate effect on blood vessels, smooth muscles, and glandular secretion, followed a few hours later by cellular infiltration at the involved site (Sampson, 1999). This type of inflammatory reaction is commonly referred as immediate hypersensitivity (Fig 1.2). It may manifest in many ways, depending on the target organ or tissue (skin, gastrointestinal system, respiratory system or generalized), and may range from life-threatening anaphylactic reaction to the lesser annoyances of atopic allergies viz., hay fever, allergic rhinitis, asthma and food allergy.

### 1.3.3. Allergic sensitization and reaction

In an allergen sensitized subject with atopy, exposure of skin, nose, or airways to a single dose of allergen produces cutaneous wheal-and-flare reaction, sneezing and runny nose, or wheezing within minutes (Table 1.4), respectively (Helm and Burks, 2000; Kay, 2004). Depending on the amount of allergen, the immediate hypersensitivity reaction is followed by a late phase reaction, this reaches a peak 6-9 h after exposure to allergen and then slowly resolves. In the skin (Fig 1.3), late phase reactions are characterized by an edematous, red, and slightly indurated swelling; in the nose, by sustained blockage; and in the lung by further wheezing (Kay, 2002; 2004).

I.

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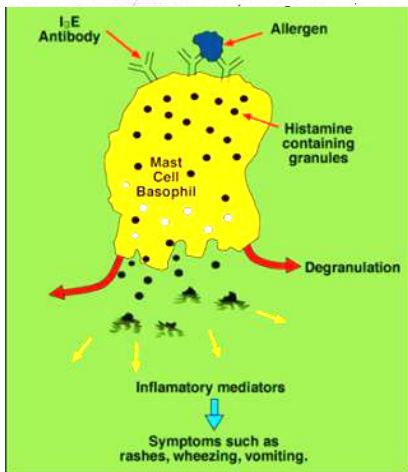


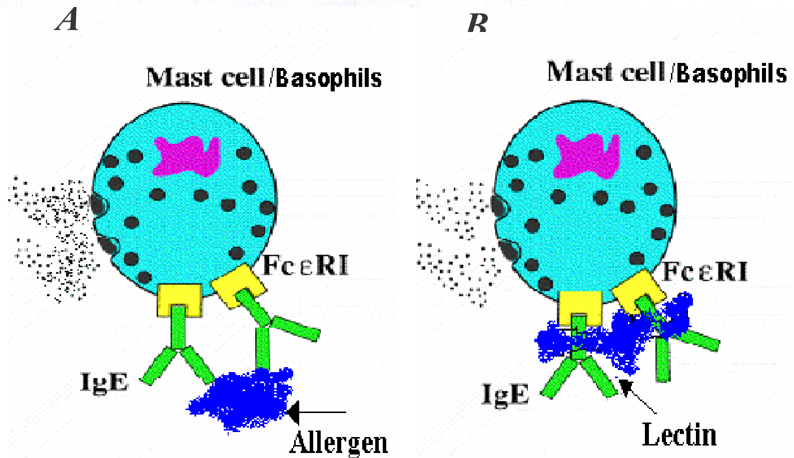




*Fig. 1.2. I. Enhanced IgE dependent effector function and potential immunoregulatory function in mast cells or basophils after IgE-dependent upregulation of FcεRI surface expression (Wedemeyer et al., 2000). II. Mechanism that triggers the degranulation of mast cell or basophils. Crosslinkage can be mediated by: (A) the allergen that initiated the IgE response, or (B) by a lectin that recognizes carbohydrates on Fc part of the IgE molecule. [www.google/allergy mechanism; accessed on Dec 2006].*

A strict relationship between genetic, skin behavior, immunological factors and trigger events such as environmental, psychological, and infections may be elicited and considered to be involved in the development and severity of allergy.

**Table 1.4. Classification of IgE-mediated allergic reactions**

A. Immediate (early-phase reaction)

- Gastrointestinal
- Hives, angioedema
- Rhinitis, asthma
- Anaphylaxis

B. Immediate (late-phase reaction)

- Eczema/atopic dermatitis
- Eosinophilic gastroenteritis
- Urticaria

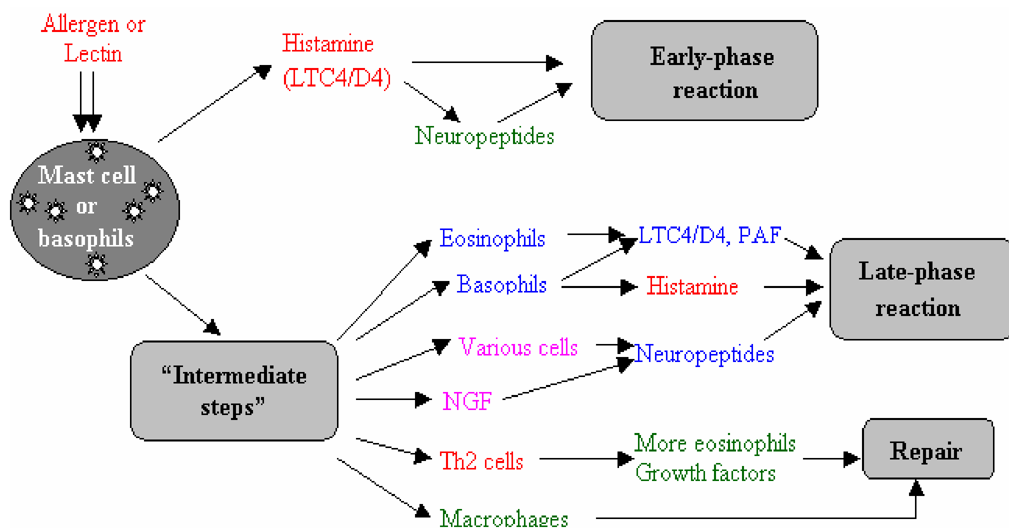



*Fig. 1.3. The progression of allergic inflammation (e.g. in skin on testing), shows the schematic representation and components involved in the development of early-phase and late-phase reactions (Kay, 2002)*

### 1.3.3.2. Mechanism of IgE-mediated (type I) hypersensitivity (allergy)

Cross-linking of bound IgE by antigen is thought to activate a guanosine triphosphate (GTP)-binding protein that, in turn, causes the activation of a phosphatidyl inositol bisphosphate ($PIP_2$)-specific phospholipase C. This enzyme, catalyse release of inositol triphosphate ($IP_3$) and diacylglycerol (DAG) from membrane $PIP_2$. $IP_3$ causes

release of intracellular calcium ($Ca^{2+}$) from the endoplasmic reticulum (Yamaguchi et al., 1997; Turner and Kinet, 1999). $Ca^{2+}$ in the cytoplasm directly activates certain enzymes, such as phospholipase $A_2$, and, in complex with calmodulin, activates other enzymes such as myosin light chain kinase. $Ca^{2+}$ and DAG combine with membrane phospholipids to activate protein kinase C (PKC). These intracellular events in mast cells or basophils result in the generation of lipid mediators, and the exocytosis of preformed secretary granules (Turner and Kinet, 1999) (Fig 1.4).

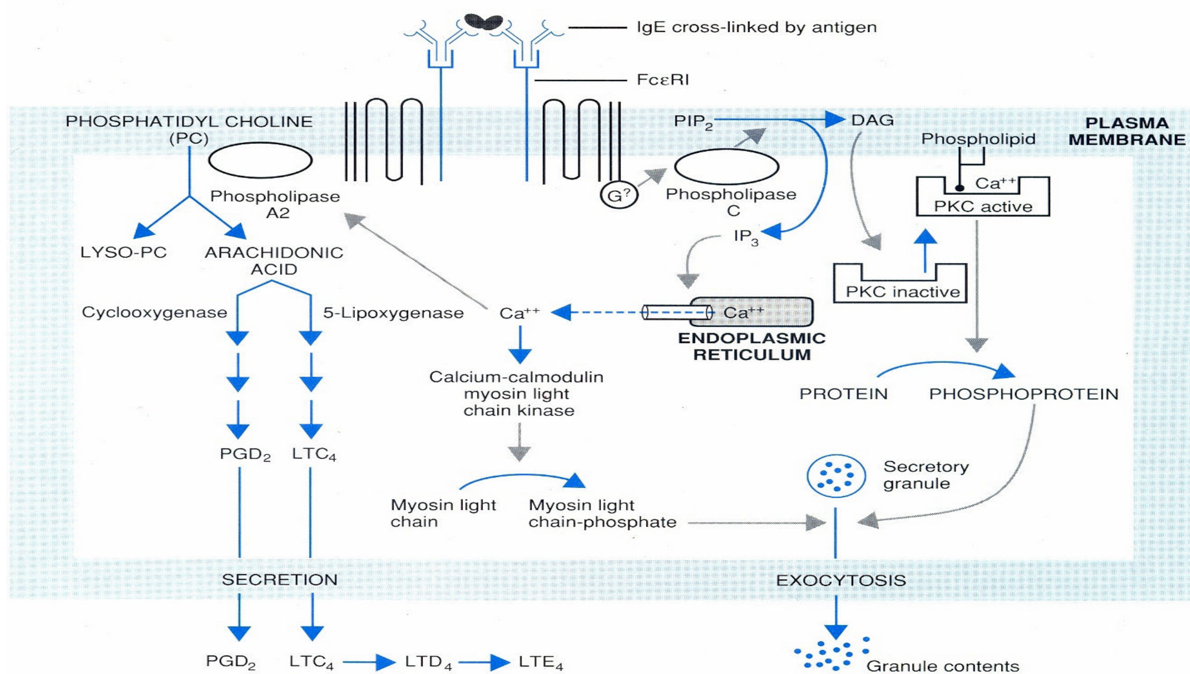



*Fig. 1.4. Biochemical events of mast cell or basophil activation (Yamaguchi et al., 1997). There is a sequence of biochemical events following the bridging of cell bound IgE by an antigen. The climax of the whole process is the non-cytotoxic secretion of various chemical mediators such as histamine and other pharmacological substances (taken from Abbas et al., 1991).*

### 1.3.5. Modes of activation of mast cells/basophils by allergen, anti-IgE and lectin

The bridging of adjacent cell-bound IgE or FcεRI by an antigen induces activation and degranulation of mast cell or basophil. The bridging may be possible with a molecule that has the ability to bind IgE or directly to FcεRI. Antigens can bind to $(Fab)_2$

of IgE whereas the anti-IgE antibody can bind to Fc portion of IgE, and anti-FcεRI can directly cross-link adjacent FcεRI. The dietary lectin that recognizes the carbohydrates on the Fc part of the IgE molecule or on the glycans of α-subunit of FcεRI can also cross- link and induce the activation of basophils or mast cells (Kjaer and Frokiaer, 2005). The modes of activation by allergen, anti-IgE and lectin are shown in Fig. 1.5.

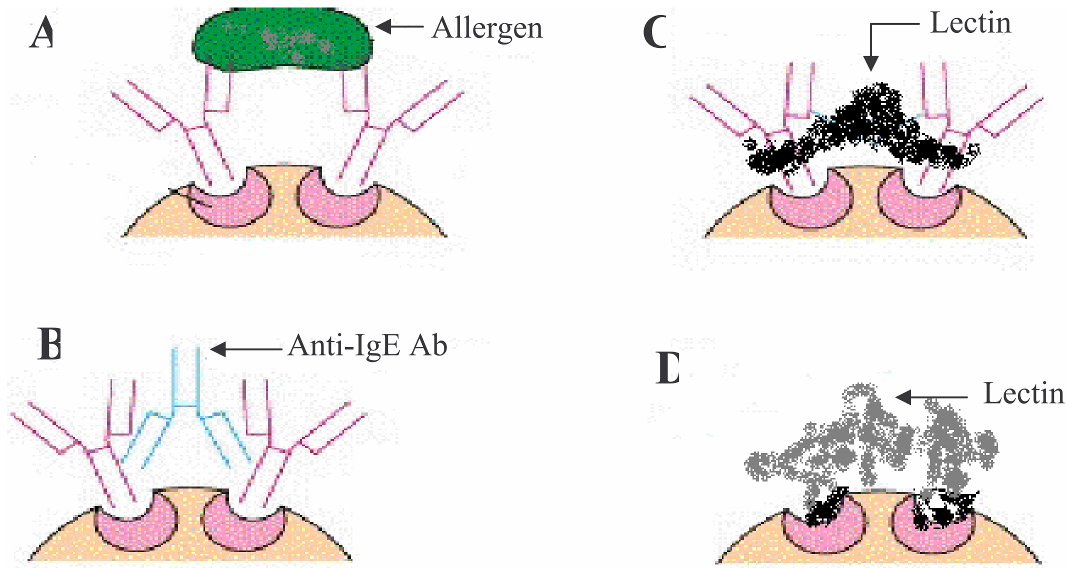



*Fig. 1.5. Schematic diagram of mechanisms that can trigger degranulation of mast cells or basophils. Cross-linkage can be mediated by the allergen (A), by an anti-IgE antibody (B), or by a lectin that recognizes carbohydrates on IgE (C) and on FcεRI (D) (Kjaer and Frokiaer, 2005).*

### 1.3.6. Cells and components of immune system involved in allergic reactions

Many cells, which are involved in the immune function, like mast cells, basophils, Th2 cells, and eosinophils play a very important role in the development of allergic reactions. The complex of the immune cell components involving antigen, IgE and FcεRI are essential for activation and mediator release.

#### 1.3.6.1. Atopic allergens

The allergens responsible for atopic diseases are derived principally from natural and airborne organic particles, especially plant pollens, fungal spores, and animal or insect debris, and to lesser extent from ingested food. The ability of different pollens,

molds, or foods to sensitize for IgE allergy varies, so that some of these environmental allergens are intrinsically more sensitizing than others, irrespective of the amount of exposure. Some lectins have been identified as allergens [peanut agglutinin (Burks et al., 1994, soybean agglutinin (Baur et al., 1996) and wheat germ agglutinin (Weichel et al., 2006)] and are in general recognized as minor allergens in comparison with other common major allergens.

#### 1.3.6.2. Mast cells and basophils

`Mast cells are mononuclear cells with densely stained metachromatic granules while basophils are polymorphonuclear and are smaller in size (Table 1.5), approximately 5-7 microns versus 10-15 microns (Wedemeyer et al., 2000; Kay, 2002; 2004).

**Table. 1.5. Properties of human mast cells and basophils**

| Cell properties | Mast Cells | Basophils |
|---|---|---|
| Cell diameter | 10-15 $\mu$m | 5-7 $\mu$m |
| Nucleus | Bilobed or multi-lobed | Round or oval; eccentric |
| Cell surface contour | Smooth with occasional short, broad projections | Numerous narrow projections |
| Predominant localization | Connective tissues | Blood |
| Life span | Weeks or months | Days |
| Terminally differentiated | No | Yes |
| Major granule contents | Histamine, chondroitin sulphate, neutral proteinases, tryptase, heparin, TNF$\alpha$ | Histamine, chondroitin sulfate, neutral proteinases, major basic protein, Charcot-leyden protein |
| Mediators that are synthesized and released after degranulation | TNF$\alpha$, PAF, $LTC_4$, $PGD_2$, IL-4 | $LTC_4$ |

Abbreviations: TNF-tissue necrosis factor; PAF-platelet activating factor; $LTC_4$-leukotriene $C_4$; $PGD_2$-prostaglandin $D_2$ (Wedemeyer et al., 2000; Helm & Burks, 2000).

Mast cells are distributed in essentially all body tissues, and are most often found adjacent to the microvasculature. Their growth and differentiation appear to be influenced by various cytokines including stem cell factor (c-kit ligand). In contrast, basophils originate from a distinct hematopoietic precursor under the influence of different cytokines such as interleukin-3, and in many ways, are more similar to eosinophils. They are mobile and although they represent less than 1% of the circulating leukocyte population, they may accumulate within tissue sites during allergic inflammation.

#### 1.3.6.3. FcεRI and immunoglobulin E (IgE)

The high affinity receptor (FcεRI) on mast cells and basophils consist of a complex of four subunits: one $\alpha$ chain, one $\beta$ chain, and two $\gamma$ chains ($\alpha\beta\gamma_2$). The $\alpha$ chain is needed for IgE binding, while the other two types of chains are needed for signal transduction (Garman et al., 2001). This receptor binds IgE with extremely high affinity ($10^9$ to $10^{10}$/mole).

The reaginic antibody (IgE) molecule is important for the identification of allergic hypersensitivity. IgE is a glycoprotein of 190 kD and has 12% carbohydrate by weight; it shows a wide range of serum concentrations (ng/mL to μg/mL) with an atopic serum half-life of 2-3 days and a much longer half-life in tissues (Gould et al., 2003). IgE sensitizes mast cells and basophils *in vitro* and *in vivo* and gets inactivated by heating (56°C, 30 min). It is synthesized by plasma cells induced by IL-4 and IL-13. The presence of allergen-specific IgE antibodies represents the single most determinant of allergic sensitivity.

#### 1.3.6.4. Th2 or $CD4^+$ cells

Atopic individuals, by contrast, have an exaggerated response characterized by the production of allergen-specific IgE antibodies. They have elevated serum levels of IgE. Several studies have shown that T cells from the peripheral blood respond to an allergen *in vitro* by producing cytokines of the Th2-type, i.e. IL-4, IL-5 and IL-13

(Romagnani, 1991; Till et al., 1997), rather than cytokines of Th1-type (IFNγ and IL-2). The immunopathological hallmark of allergic disease is the infiltration of affected tissues by cells with a Th2-type cytokine profile (Kay et al., 1991; Robinson et al., 1992).

**1.3.6.5. Mediators released by mast cells and basophils**

The pathologic relevance of mast cells and basophils stems largely from their ability to synthesize a diverse collection of potent chemical mediators (Kay, 2002). Mast cell or basophil mediators are commonly considered to fall into three main groups: preformed mediators that are rapidly released from the granules following activation; preformed mediators that remain granule-associated or released slowly following activation; and mediators that are synthesized *de novo* following activation, often by the metabolism of membrane phospholipids (Turner and Kinet, 1999). The complete catalog of substances that have been identified in mast cells and basophils is quite extensive. This undoubtedly account for the multiple possible consequence of allergic reactions.

**1.3.7. *In vivo* and *in vitro* diagnosis of allergic reactions**

When allergy is suspected, the diagnostic process is aimed at determining whether the disease is caused by allergy and, if so, to identify the type of allergy and each of the responsible allergens. Many straightforward cases can be diagnosed easily and quickly, but more complex or obscure allergic diseases require considerable detective work. History, physical examination, and appropriate laboratory tests are required, as in the diagnosis of any medical condition (Fig 1.6). A detailed case history is very essential. It is important to correlate the results of specific allergy testing to the patient's history. Whenever allergy is suspected, the physician should first obtain a detailed description of the symptoms and the timing and environmental locations associated with appearance and disappearance of symptoms. Other historical data such as the age and onset of illness, the influence of prior treatments, help to distinguish allergic from non-allergic conditions.

Allergic diseases are often episodic, because signs and symptoms depend on exposure to the allergen. Objective signs of allergy are therefore present when the physical examination is performed during the period of allergen exposure. The examination should be thorough enough to rule out other causes for patient's symptoms. A variety of laboratory tests are available to supplement the history and physical examination. There are procedures for quantitating the extent of functional and anatomic effects on a particular organ, for sampling fluids, tissues, or cells for evidence of disease, and for establishing the presence of specific immune response.

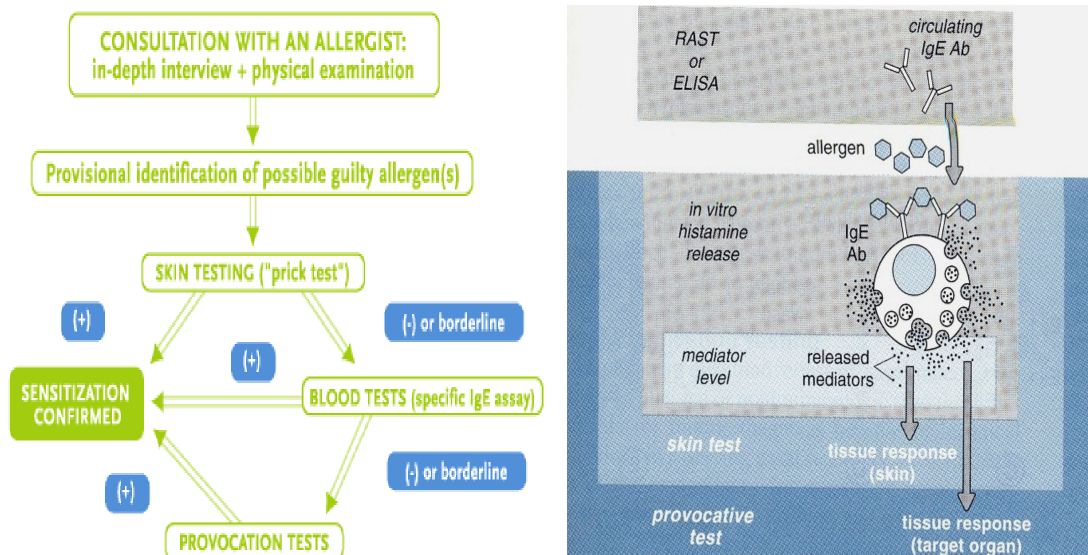



*Fig. 1.6. Schematic diagram showing the identification and assessment of atopic status using clinical history, physical examination and laboratory tests (Kay, 2002; www.google/allergy diagnosis, accessed on Jan 2007).*

**1.3.8. Dietary lectins in non-allergic food hypersensitivity**

Lectins such as Con A, LCA, PSA, RCA and PHA-E have been shown to be able to bind with high affinity to IgE from atopic subjects. They have also been shown to induce the release of histamine when leukocytes from atopic subjects were stimulated (Kjaer and Frokiaer, 2005). As dietary lectins are able to bind to human IgE, they might induce symptoms like those of allergy by cross-linking IgE molecules on mast cells or basophils, which may eventually lead to allergic or inflammatory reactions known as 'non-allergic food hypersensitivity' reactions (formerly known as false food allergy)

(Roehr et al., 2004). The dietary lectin induced degranulation of mast cells/basophils exactly mimic the food allergen-specific IgE interaction seen in food allergy, though the initial site of interaction is different.

### 1.4. Mitogenicity of plant lectins

Mitogenic agents are capable of inducing mitosis and cell division. Lectins are able to induce cell division in different kinds of cells, and as mentioned earlier, some plant lectins are mitogenic towards enterocytes. The *in vitro* mitogenicity of lectins is typically measured as their ability to induce proliferation of lymphocytes from lymph organs or blood (Fig 1.7). Lectins are used to induce proliferation in experimental immunology; the best described and most used lectins are PHA and Con A. They are used as polyclonal activators irrespective of antigenic specificity, and the frequency of responding cells is very high. PZR is a major receptor identified for con A on various cells and has an important role in cell signaling via c-src, resulting various biological activity of con A (Zhao et al., 2002). After prolonged contact with lectins, lymphocytes proliferate and become mature effector cells that secrete cytokines and may exert effector functions such as cellular toxicity and antibody production (Kilpatrick, 1999).

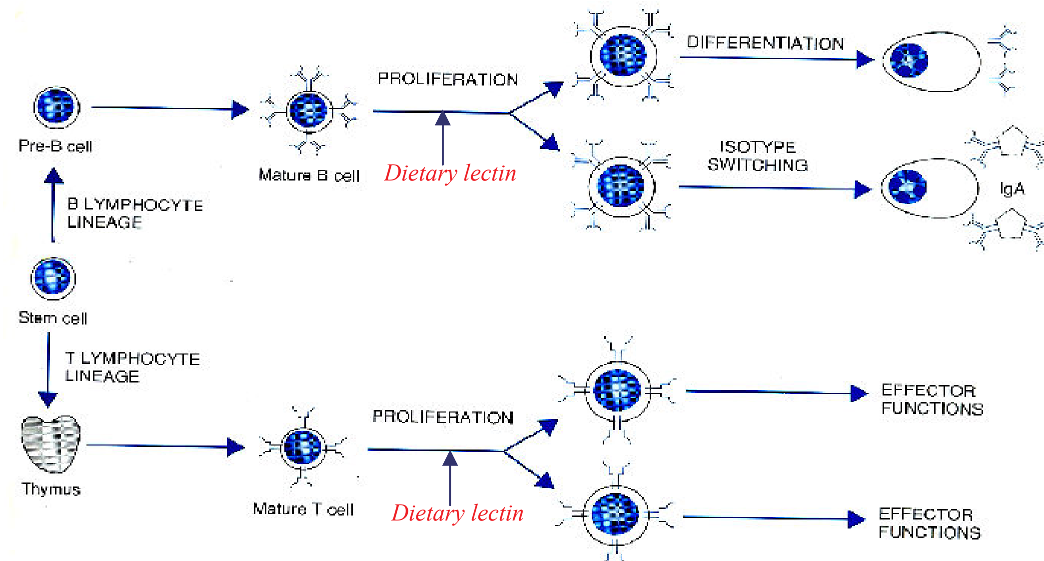



*Fig. 1.7. The mitogenic effect of dietary lectins for proliferation of T and B-cells, which may exert effector functions such as cellular toxicity and antibody production (taken from Kilpatrick, 1999; Abbas et al., 1991)*

However, not all plant lectins are mitogenic; they can be grouped as mitogenic, non-mitogenic or antimitogenic. Actually, WGA has been found to be nonmitogenic (Muraille et al., 1999), antimitogenic (Barret et al., 1983), and mitogenic for either T cells or B cells (Kilpatrick, 1995), depending on the concentration of the lectin or the purity of the examined cells. Antimitogenic lectins inhibit the performance of mitogens in co-culture experiments. Whether a lectin is mitogenic or antimitogenic might also depend on the position of the sugar moiety to which the lectin binds. If the carbohydrate to which the lectin binds is located in close proximity to the binding site of the receptor, binding of lectin does not lead to activation, but the lectin may act as an antagonist due to sterical hindrance of ligand binding.

Some of the so-called antimitogenic lectins that fall in this category are potato and tomato lectins (McCurrach and Kilpatrick, 1988). Lectins shown to be non-mitogenic may also influence cells of the immune system. For example, WGA has been found to be non-mitogenic, but at the same time the lectin was able to induce secretion of IL-12 and IFN$\gamma$ (Muraille et al., 1999). The mitogenicity of a lectin can differ depending on the animal from which the cells originate, as well as the immune compartment (Fronkiaer et al., 1997). The variation in cell responsiveness towards different lectins is not surprising, as cell surface saccharides change during development and differentiation and cells from different immune compartments are at different developmental stages. It has been shown that binding of some lectins to the T-cell receptor induces mitogenesis (Chilson and Kelly-Chilson, 1989). Reviews on the mitogenic action of lectins have appeared (Heegaard and Muller, 1988, Kilpatrick, 1999), however, knowledge of the mechanism by which lectins induce mitogenisis remains in its infancy.

**1.5. Mucosal immunogenicity of ingested dietary lectins**

Relatively few molecules have been identified that are able to induce a strong immune response when delivered by the oral or other mucosal routes. Lectins are some of the few proteins that when administered by the mucosal route induces an antibody response. Although still sparsely documented, the type of immune response (local vs.

systemic, tolerance vs. immunity) may be strongly dependent on the site of absorption in the gut. Uptake across Peyer's patches (PP) might induce immunity, whereas, absorption through enterocytes might induce tolerance. Apart from indications that the binding activity of lectins can confer immunogenicity, there are very few investigations performed on what determines mucosal immunogenicity of plant lectins.

### 1.5.1. Plant lectins with mucosal immunogenicity

Many different plant lectins have been shown to be mucosal immunogens in rodents (Di Aizpurua and Rossell-Jones, 1988). In humans, banana lectin was found to induce a strong specific antibody response, especially of the $IgG_4$ isotype (Koshte et al., 1992). PHA is highly immunogenic orally; in fact, orally applied PHA mounted an antibody response to the same level as seen in parenteral administration (Di Aizpurua and Rossell-Jones, 1988; Kjaer and Frokiaer, 2002, 2005).

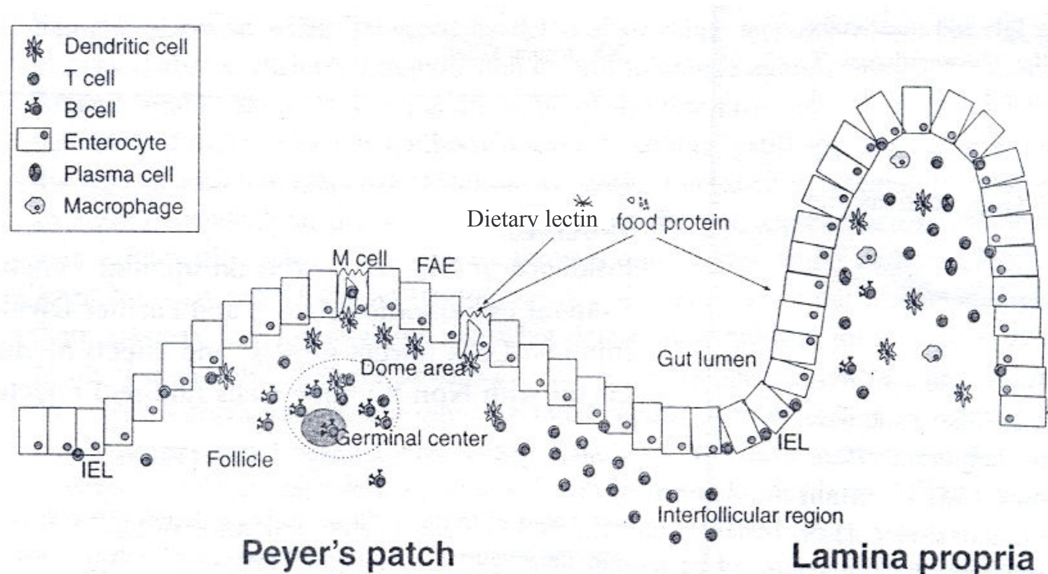



*Fig. 1.8. Schematic representation of the general structure and major cell populations of the gut-associated lymphoid tissue (GALT), which is composed of Peyer's patches, intraepithelial cells (IEL), and cells of lamina propria. M cells are present in the follicle-associated epithelium (FAE). Dietary lectins are able to bind to glycans on the luminal side of enterocytes or M cells; binding destroys the epithelium and results in enhanced uptake of lectins and other luminal co-administered antigens (food proteins) (Kjaer and Frokiaer, 2005).*

An increase of intraepithelial lymphocytes and jejunal lamina propria cells was observed in response to orally administered PHA (Banwell et al., 1993), suggesting that absorption of PHA leads to T-cell proliferation in gut-associated lymphoid tissue (GALT).

### 1.5.2. Generation of specific anti-lectin immune response

Lectins are highly immunogenic and capable of inducing a specific immune response after oral administration, in contrast to other dietary proteins. Orally ingested lectins may, after absorption, affect the immune system systemically; however, it is also important to consider the local mucosally induced immune response, as the mucosa is where orally ingested lectins initially encounter the immune system. Natural serum antibodies (for ASA I and alliinase present in garlic) against dietary proteins have been detected in humans (Tchernychev and Wilchek, 1996) and various lectins are capable of inducing a specific IgG response in mice (Kjaer and Frokiaer, 2002). Lavelle et al. (2000) have investigated the mucosal and systemic immune response to a number of mucosally administered plant lectins. VAA-I showed the highest mucosal specific immune response. None of the lectins examined, viz., PHA, WGA, UEA-I, LEA and VAA-I gave rise to a strong mucosal response in the absence of systemic response (Lavelle et al., 2000). WGA and UEA-I induced a stronger response when administered by the oral route as compared to intranasal administration (Lavelle et al., 2000).

### 1.5.3. Adjuvant activity of dietary lectins to the co-administered antigens

Dietary lectins may influence mucosal immunity against other co-administered proteins, i.e., display adjuvant activity; co-administration of lectin with other proteins leads, in many cases, to an immune response against the co-administered protein. Con A complexed to ovalbumin (OVA) has been shown to enhance the formation of anti-OVA IgG and IgE in mice upon immunization (Gollapudi and Kind, 1975). Nasal and subcutaneous administration of Con A has been found to induce IgE antibodies against Con A and against hapten-conjugated Con A (Mitchell and Clarke, 1979). PHA has been reported to affect the OVA-specific IgE response in mice injected with OVA and

PHA (Astorquiza and Sayago, 1984). More recent studies have also demonstrated that lectins affect the immune response against mucosally-administered OVA (Lavelle et al., 2001; Watzl et al., 2001). Plant lectins are, thus, not only mucosally immunogenic, but also have mucosal adjuvant activity, as tested by co-administration of OVA (a weak antigen) and different lectins (Lavelle et al., 2001).

#### 1.5.4. Role of dietary lectins in the onset of allergy

Oral ingestion of jackfruit seed lectin (jacalin) provoked an enhanced IgE response towards both OVA and the lectin after parenteral challenge (Restum-Miguel and Prouvost-Danon, 1985). Many plant lectins are known to induce IL-4 and IL-13, which promote the differentiation of T helper cells towards Th2 cells (Abbas et al., 1996; Finkelman et al., 1990). Lavelle et al. (2000) have found that mucosal administration of PHA, WGA and UAE-I gave rise to IgG1 antibodies indicative of a Th2 response in BALB/c mice. Intranasal immunization with LEA and VAA-I induced lectin-specific antibodies of the IgG1, IgG2a, IgG2b and IgA isotypes, whereas PHA induced only IgG1 and IgA in serum (Lavelle et al., 2000; 2001). An important outcome of their study was, when using isotype antibodies as markers of Th1/Th2 immunity, that lectins are more Th2-skewing toward a Th2 response than is cholera toxin (Lavelle et al., 2000), which is known to be a potent Th2-skewing immunogen (Xu-Amano et al., 1993). The ability of lectins to promote polarization is relevant in connection with certain diseases, such as allergy, which is a Th2-driven disease.

### 1.6. Interaction of dietary lectins with immune function

Lectins may interact with the immune system in various ways. Dietary lectins may lead to generation of a specific anti-lectin response, but may also modulate immune response against co-administrated proteins, the so-called adjuvant effect. Moreover, lectins may also be capable of polarizing the immune response towards certain effector functions (Table 1.6). In addition, dietary lectins may give rise to mucosal (local) or systemic response (Kjaer and Frokiaer, 2005).

A number of plant lectins have been found to be stable in the rodent gut and to interact with the mucosal epithelium after feeding (Pusztai and Bardocz, 1996). There is recent evidence for the translocation of plant lectins across the gut in both mice and humans (Clark et al., 1995; Wang et al., 1998). The finding that certain plant lectins interact with the mucosal epithelium and are translocated across the gut may have important applications in inducing mucosal and systemic immunity.

**Table. 1.6. Influence of dietary lectins on immune function: putative systemic and mucosal (local) immune responses**

| Immune response | Generation of lectin-specific response | Immunomodulating effects |
|---|---|---|
| Systemic response | Specific IgG, IgM and IgE antibodies | Adjuvant activity (immune response as opposed to tolerance)<br>Polarizing effect (Th1 or Th2 polarization)<br>Release of histamine and other bioactive mediators (allergy symptoms)<br>Increase in NK cell activity |
| Mucosal response | Specific IgA antibodies | Mucosal adjuvant activity (immune response as opposed to tolerance)<br>Release of histamine and other bioactive mediators (allergy symptoms) |

Dietary proteins usually give rise to specific downregulation of the immune response, due to induction of oral tolerance. Ingested lectins initially encounter the immune system and various plant lectins are capable of inducing a specific IgG response in mice (Di Aizpurua and Russel-Jones, 1988, Hjaer and Frokiaer, 2002, 2005). LEA and PHA showed high immunogenicity after nasal administration; LEA also gave rise to specific IgA and IgG responses (Lavelle et al., 2000). High level of specific serum IgG was induced by oral administration of LEA to mice (Naisbett and Woodley, 1995), whereas nasally administered PHA only demonstrated an IgA response systemically and not mucosally (Lavelle et al., 2001).

Some of the earlier studies have shown that proteins with lectin/lectin-like properties are effective mucosal immunogens, and proposed a relationship between receptor binding in the gut and mucosal immunogenicity (De Aizpurua and Russell-Jones, 1988). However, despite a number of studies on lectin binding, and evidence that plant lectins conjugated to antigens/haptens may enhance immune responses following oral (De Aizpurua and Russell-Jones, 1988) and nasal (Giannasca et al., 1997) delivery, there is relatively little data on the comparative mucosal immunogenicity of plant lectins (Table 1.7).

**Table. 1.7. Influence of dietary lectins on immune function: specific and general immunomodulation**

| Parameter | Direct effects (lectin interaction with the immune cells) | Indirect effects (lectin interaction with non-immune cells) |
|---|---|---|
| Generation of lectin-specific immune response | Cross-binding of lectins to B-cells (T cell-independent antibody response, mitogenesis) | Binding of lectins to red blood cells (particulate antigen)<br>Binding of lectins to enterocytes (increased absorption) |
| Immunomodulation | Cross-binding of Fc receptors on mast cells (histamine release)<br>Binding to APCs or T cells (mitogenesis, polarization and adjuvant effects)<br>Increase in NK cell activity (increased cytotoxicity) | Intestinal degeneration (increased and/or altered absorption) |

The immune responses against dietary lectins thus seem to be natural consequence of lectin ingestion, but whether this, in any way, has an adverse influence on the immune system is not fully elucidated. Also, in light of the intensive research in use of lectins as drug and vaccine delivery systems and as potential antileukemia agents (Gabor and Wirth, 2003; Lavelle, 2001), the immune response to orally ingested lectins needs further research.

**Aim and focus of the present study**

Studies on the interaction of dietary lectins with cells of the immune system are rather limited in literature. The interaction of lectins with basophils and mast cells releases histamine and other biological mediators have been studied with only a few lectins thus, is in many respects similar to the interaction of food allergens with mast cells/basophils from allergic subjects. Allergic reactions caused by the activation of mast cells/basophils by lectins present in food sources termed as non-allergic food hypersensitivity reactions (commonly known as false food allergy). Dietary lectins can either interact with carbohydrates on cell-bound IgE or directly on with the carbohydrates of cell surface glycoproteins or glycolipids on basophils or mast cells. In both these cases, the end results are similar to the food allergen-specific IgE interaction seen in food allergy Therefore it is important to address the role of plant dietary lectins in mediating non-allergic food hypersensitivity reactions.

Lectins may interfere in with the immune system in various ways. Dietary lectins can lead to the generation of anti-lectin response, but they may also modulate immune response against co-administered proteins, so called adjuvant effect. Moreover, lectins may also be able to polarize the immune response towards certain effector function. In addition, dietary lectins may give rise to a mucosal (local) or a systemic response which shows the influence of dietary lectins on immune function. Another way of categorizing immunomodulatory effects is the *in vitro* mitogenicity of lectins which is typically measured as their ability to induce proliferation of lymphocytes and lymph organs or blood. Lectins are used to induce proliferation in experimental immunology.

The present study is focused on the modulatory effects of certain purified dietary lectins on the cells of the immune system (basophils, mast cells and lymphocytes), which will aid us to delineating the role of lectins in non-immune mediated food allergic reactions (false-food allergy) and their modulatory effects on immune cells to induce immunogenicity. Since lectins are often the major proteins in many plant foods and are found to interact with the surface glycans of many cellular systems it is interesting to

study the modulatory aspects of certain dietary lectins to understand their importance and role in relation to hypersensitivity and immunogenicity.

The lectins selected for study have following specificities: Poly-(N-acetyl glucosamine) specific potato lectin (Solanum tuberosum agglutinin; STA), N-acetyl galactosamine specific horse gram lectin (Dolichos biflorus agglutinin; DBA) and Mannose specific garlic agglutinins (Allium sativum agglutinins; ASA I and ASA II). Potato lectin and garlic agglutinins were isolated in the laboratory by published procedures using classical protein purification techniques, whereas purified Dolichos biflorus agglutinin were procured from commercial sources. The present study focuses on the following objectives with an interest of study the modulatory aspects of these dietary lectins with the immune cells involved in hypersensitivity and immunogenicity.

1) To investigate the effect of certain purified lectins on cells of the immune system involved in allergic reactions (specifically on basophils and mast cells) with a view to understand their role in non-immune mediated food allergic reactions (false food allergy).
2) To study the immunomodulatory effect of purified proteins (native and heat-processed potato lectin, and garlic agglutinins I, II) on human and murine lymphocytes.
3) To study the mucosal adjuvant effects of garlic lectins (*Allium sativum* agglutinins I and II) in order to assess the immunogenicity of these lectins.

The main focus of the present study is to provide valuable information on how dietary lectins with different carbohydrate specificity activate the effector immune functions with the cells of immune system. This helps us to delineate the role of dietary lectins with the cells of immune system, which are involved in hypersensitivity and immunogenicity.

# Chapter 2

# Effect of horse gram lectin (*Dolichos biflorus* agglutinin) on degranulation of mast cells and basophils of atopic subjects

## 2.1. INTRODUCTION

Allergy is the clinical manifestations of immunologically mediated reactions to allergens in atopic (sensitized) individuals. The allergens identified so far are, in general, proteins or glycoproteins of molecular mass 10-70 kD. The sources of allergens are extremely diverse and cover much of the biosystems to which man is exposed, for example, inhalant allergens (pollens, house dust mite), contact allergens (chemicals) and ingestant allergens (food and drugs). While food is essential for life, it also represents the largest antigenic load faced by human immune system (Sampson, 2004). Up to 8% of children of age below 3 years and approximately 3% of the adult population experience food-induced allergic disorders (Walker-Smith, 2005).

Immediate hypersensitivity is the basis of acute allergic reactions caused by the activation of basophils and mast cells when an allergen interacts with membrane-bound IgE (Kay, 2002). The complex of allergen, IgE, and FcεRI on the surface of these cells triggers the release of histamine and other biological mediators (Schwartz, 2004; Galli et al., 2005). Collectively, these mediators are responsible for the clinical symptoms seen in a variety of allergic disorders. Another mode of activation occurs when some plant lectins cross-link two adjacent IgE molecules, by binding to the carbohydrates on the Fc portion of IgE (Margo, 1974; Haas et al., 1999). Con A, which has specificity for mannose/glucose, was the first lectin shown to activate basophils and mast cells (Margo. 1974; Siraganian and Siraganian, 1974; 1975). Other lectins studied in this regard are mostly mannose- and GlcNAc oligomer-specific lectins (Van Damme et al., 1998; Shibasaki et al., 1992). Lectins are an important component of total dietary proteins in foods, especially in the case of legumes where they are present in amounts of up to 25% (Etzler, 1997; 1998).

Horse gram (*Dolichos biflorus*) plant is a native of India and is distributed throughout the tropical regions of the old world. It occurs all over India up to an altitude of 5,000 ft. It is an important pulse crop particularly in Chennai, Mysore, Mumbai and Hyderabad (CSIR, 1952). Horse gram is grown in almost all states as dry crop on any type of soil except highly alkaline types. It grows on poor soils and is hardy and drought-

resistant. Several varieties of horse gram differing in the color of seed coat and the period of maturity are known under the cultivation. The cultivated crop is usually a mixture of several varieties.

Horse gram is extensively used in south India as feed for cattle and horses. The seeds are cooked before feeding. Stems, leaves and split husk are also used as cattle feed. Horse gram seeds are consumed widely, after cooking or frying, among rural populations compared to the urban. The seeds are germinated and are used in preparation of foodstuffs like curry and rasam. They are also eaten whole or after grinding into a meal, unlike other pulses, which are consumed after splitting (Kadam and Salunkhe, 1985). It is a major pulse used for consumption in the rural sector among poor classes. Horse gram is a valuable protein supplement. The composition of the seeds is as follows: 11.85% moisture, 57.3% carbohydrate, 22.0% crude protein, 0.55% fat, 3.15% mineral matter and 5.3% fiber (Gopalan et al., 2000).

The leguminous plant, horse gram contains a seed lectin, which constitutes up to 10% of the soluble protein of the seed cotyledons (Talbot and Etzler, 1978). Horse gram lectin (*Dolichos biflorus* agglutinin; DBA) agglutinates type $A_1$ erythrocytes and has specificity for terminal $\alpha$-linked *N*-acetyl-D-galactosamine (GalNAc) (Etzler, 1997; 1998).

It is a heterotetrameric glycoprotein found in two forms, A and B: form A (113 kD) and form B (109 kD). The predominant form A is composed of four similar subunits, which are grouped into two subunits, IA (27.7 kD) and IIA (27.3 kD). There are two GalNAc-binding sites per lectin molecule, and both are present on subunit IA (Etzler, 1997; 1998).

The lectin exhibits a high degree of binding specificity for terminal non-reducing $\alpha$-linked N-acetylglucosamine residues with an apparent binding stoichiometry of two sugar molecules per tetramer (Etzler et al., 1981). Structural data on the seed lectin indicate that the subunit types are nearly identical, with the difference detectable only at their carboxyl-terminal ends (Roberts et al., 1982). The studies on the biosynthesis of the horse gram lectin have shown that both subunit types arise from a single polypeptide

precursor. The difference between the subunits apparently arises from specific post-translational proteolytic processing of the precursor molecule (Schnell and Etzler, 1987).

Apart from mannose- and GlcNAc oligomer-specific lectins (Margo, 1974; Siraganian and Siraganian, 1974; 1975; Shibasaki et al., 1992), lectins with other sugar specificities have not been studied in detail with respect to their effects on basophils and mast cells. It appears that some dietary lectins are responsible for non-allergic food hypersensitivity reactions that exactly mimic the symptoms of immediate or type I (IgE-mediated) hypersensitivity reactions (Shibasaki et al., 1992). In view of the extensive consumption of horse gram in the Indian sub-continent, it appeared interesting to study the effect of purified horse gram lectin on the degranulation of mast cells *in vivo* and basophils *in vitro* from non-atopic and atopic subjects.

## 2.2. MATERIALS

This study was undertaken after clearance by the Institutional Ethics Committee; informed consent was obtained from all atopic and non-atopic subjects in the age range of 15-60 years. Male Wistar rats housed in the animal house facility of this institute was used for the preparation of peritoneal exudate cells (PEC) as per standard operating procedures, after obtaining approval from the Institutional Animal Ethics Committee (IAEC).

DBA (*Dolichos biflorus* agglutinin; lyophilized powder, agglutination activity <20 μg per mL, purified by affinity chromatography), compound 48/80, concanavalin (con A), murine anti-human IgE (monoclonal)-alkaline phosphatase (AP) conjugate were products of Sigma-Aldrich Co., St. Louis, MO, USA. Lysozyme, ovalbumin (OVA), and bovine serum albumin (BSA) were purchased from Bangalore Genei, Bangalore, India. Dextran T 700, *o*-pthalaldehyde (OPT) were products of Hi-Media Laboratories, Mumbai, India. Flat-bottom 96-well microtiter plates (MICROLON) were bought from Greiner Bio-One GmbH, Frickenhausen, Germany. All other chemicals/reagents used in this study were of analytical grade.

## 2.3. METHODS

### 2.3.1. Identification of atopic and non-atopic subjects

These subjects were identified based on case history (atopic subjects are chosen at random who had symptoms of at least one allergic condition such as allergic rhinitis, atopic dermatitis, asthma, food allergy, and allergic conjunctivitis) of the subjects and skin prick tests (SPT) of certain commercial pollen and house dust mite (HDM) extracts. Most of the subjects examined in this study were from the rural areas. SPT was performed using various commercially available allergen extracts such as grass mix 1, grass mix 2, (pollen extracts from various kinds of grasses which contain mixture of allergens), weed mix (pollen extracts from different weeds) and house dust mite (HDM) (which contain the feces of the dust mite) that is allergic to 20% of the total atopic subjects. Grass pollen mix extract 1 was obtained from Bayer Corp., Spokane, WA, USA, and Greer Laboratories, Lenoir, NC, USA. Southern grass pollen mix (#165, Bayer) contained pollens from Bermuda, Johnson, Kentucky Blue, Orchard, Redtop, Sweet Vernal and timothy grasses. Grass pollen mix 2 (#P28, Greer Laboratories) contained pollen from Bermuda, Johnson, Kentucky Blue, orchard, redtop, timothy, sweet vernal meadow, fescue, and perennial rye grasses.

Self-reported allergic reactions after intake of certain fruits and vegetables as well as symptoms in the pollen season (asthma and rhino-conjunctivitis) were recorded by a questionnaire. SPT of various common commercial allergenic extracts such as grass mix, weed mix, and house dust mite were tested. The allergen extracts were tested with the positive control (histamine base, 1 mg/mL) and negative control (glycerinated phosphate-buffered saline). The diameter of the wheal was read 20 minutes after the skin prick. If the wheal diameter is at least 3 mm greater than that of the negative control, the test is considered positive. Subjects showing a wheal diameter from three-fourths to as much as the positive control were selected as atopic subjects for this research study.

### 2.3.2. Eosinophil count, serum total IgE levels, serum and plasma histamine levels

#### 2.3.2.1. Absolute eosinophil counts in the blood of atopic and non-atopic subjects

It is important to carry out a direct total or absolute eosinophil count to get an accurate value of total eosinophil count (Weller et al., 2003). For determination of absolute eosinophil count, 380 μL of diluting fluid was taken. [The diluting fluid used should stain the eosinophils and lyse the red blood cells; commonly used diluting fluid was Hingleman's solution that contains yellow eosin (0.5 g), 95% phenol (0.5 mL), 40% formalin (0.5 mL) and distilled water 99.0 mL]. To this, 20 μL of blood was added and mixed. The sample was then kept at 25°C for 10-15 min before charging to the hemocytometer counting chambers. Alternatively, the counting chamber may be filled immediately to prevent clumping of eosinophils and kept in a moist chamber for 5 min in a petridish for cells to settle and lyse RBC. The eosinophils were counted in all the 9 squares with low power (10 X) eyepiece. The following formulae is used for the calculation:

$$\text{Total number of eosinophils} = \frac{(\text{no. of cells counted}) \times (\text{dilution factor}) \times (\text{depth factor})}{\text{no. of areas counted}}$$

$$= \frac{z^* \times 20 \times 10}{9} = \text{eosinophils} / \mu\text{L}.$$

(* corresponds to the number of cells counted)

For a reasonably accurate count, at least 100 cells must be counted and lower counts are obtained if there is no proper filling of the counting chambers. The normal value of eosinophils is 40-400 cells / μL in healthy individuals.

#### 2.3.2.2. Serum separation from the subjects' blood

Ten milliliters of venous blood was drawn from the subject into a clean sterilized 15 mL polystyrene tube. The tube was plugged with cotton and allowed to stand at room temperature for 1 h. After clotting, it was kept at 4°C for 2 h and then centrifuged at 750 x g at room temperature for 10 min. The separated serum was aspirated and stored in aliquots at -20°C until analysis.

#### 2.3.2.3. Determination of total IgE levels in serum

Total IgE levels in atopic and non-atopic subjects was detected by enzyme-linked immunosorbant assay (ELISA) using anti-human IgE antibodies (Hamilton and Adkinson Jr, 2003) (murine IgG2a, κ; hybridoma cell line ATCC HB-121, designation E5BB3IIA2, obtained from National Centre for Cell Science (NCCS), Ganeshkhind, Pune, India) which was purified from hybridoma cell culture supernatant on protein A-agarose column affinity chromatography. For the total IgE determination, known concentration of monoclonal murine IgG2a (kappa) that reacts with human IgE (ATCC, 1985) was coated (100 μL/well) on microtiter plate coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) at $4^0$C overnight. After washing using phosphate-buffered saline (PBS) containing 0.05% Tween-20, abbreviated as PBS-T, blocking reagent (3% gelatin prepared in PBS) was added and plate was incubated at 37 $^0$C for 1 h. Then serum samples (100 μL/well) from non-atopic or atopic subjects, appropriately diluted (1:3) with diluent buffer (PBS containing 1% BSA and 0.5% Tween-20) were added to each well. The plate was then incubated in a humidified chamber at 37°C for 2 h. After incubation, 100 μL of secondary antibody [murine anti-human IgE antibody conjugated with horseradish peroxidase at a dilution of 1:2000 in diluting buffer (PBS containing 1% BSA, 0.5% gelatin and 0.05% Tween-20)] was added and the plate was incubated at 37°C for 2 h in humidified chamber. Finally, 100 μL of freshly prepared horseradish peroxidase substrate solution (0.5 mg/mL *o*-phenylenediamine and 0.006% $H_2O_2$ in 0.2 M potassium phosphate buffer, pH 7.0) was added and allowed to stand at 25 °C in the dark for 20 min. The enzyme reaction was terminated by the addition of 100 μL of 2 N HCl, and the absorbance read within 15 min at 492 nm in a ELISA reader. Values are expressed as ELISA units with one ELISA unit defined as one absorbance unit at 492 nm.

#### 2.3.2.4. Histamine quantitation assay

Histamine was quantitated by fluorometric assay using OPT as a fluorescent reagent. Histamine stock (10 mg/mL) was prepared by weighing 16.2 mg of histamine dihydrochloride, which corresponds to 10 mg of histamine base in water. The histamine

standards were prepared in the concentration range of 0-50 ng in the volume range 0-1000 μL.

The histamine content in the serum or plasma was quantitated following an extraction method (Oguri & Yoneya, 2002). Briefly, 100 μL of the serum or plasma (after treatment with 10% TCA for protein precipitation) was diluted to 1 mL using PBS. The supernatant containing histamine and other low mol. wt. mediators were extracted by manual extraction method using *n*-butanol-heptane system into 0.1 M HCl. The tubes containing histamine were made upto 1 mL using 0.1 N HCl and transferred to an ice bath and to each tube, 0.3 mL of 1 N NaOH was added and immediately 75 μL of 0.2% OPT solution was added and mixed. The reaction was allowed to proceed for 40 min in an ice bath. Then, 0.15 mL of 2 M $H_3PO_4$ was added, mixed and kept at room temperature for 15 min. The samples were read in a spectrofluorophotometer (Ex λ: 360 nm; Em λ: 450 nm).

**2.3.3. Assessment of purity of DBA by SDS-PAGE and RP-HPLC**

SDS-PAGE (12% reducing) was carried out for the commercially procured *Dolichos biflorus* lectin along with con A and marker proteins [containing BSA (66 kD), ovalbumin (46 kD) and lysozyme (14 kD)]. After the electrophoretic run, the gel was stained with either Coomassie or silver stain. Reverse phase HPLC analysis was carried out using a $C_{18}$ column (4.5 x 250 mm; particle size 5 μm) in a Shimadzu LC-10A HPLC system (Shimadzu Corp., Kyoto, Japan). The sample was eluted using a binary gradient of solvent A (0.1% TFA) and B (70% acetonitrile in 0.05% TFA) at a flow rate of 1 mL/min. Protein detection was monitored at both 230 and 280 nm. The gradient was extended in the range of 50% to 80% acetonitrile to get better resolution.

**2.3.4. Skin prick test (SPT)**

Most purified allergens (natural or recombinant) have been used for SPT in the concentration range of 20 μg/mL to 1 mg/mL. Since DBA represents 10% of the total proteins in horse gram, the DBA concentration for SPT was orbitorily chosen at 100

μg/mL. Equal volume of DBA solution in PBS (200 μg/mL) and glycerol were mixed to obtain DBA solution of 100 μg/mL. 50% Glycerinated phosphate-buffered saline was used as negative control and histamine base at 1 mg/mL (equivalent to 1.62 mg/mL of histamine dihydrochloride) was used as positive control.

Skin prick test was done following the standard protocol (Sanico et al., 2002). Initially, the forearm was cleaned by wiping with 70% ethanol and allowed to dry for few minutes. Then the sample numbers were marked and the samples were spotted using sterilized glass rods along with positive and negative controls. Each sample was pricked with a separate sterile lancet to avoid carry-over of the sample. After pricking, the SPT reaction was allowed for 20 min without any disturbance. The itching felt during the SPT for the samples were recorded. The wheal and flare diameters was read using a special ruler after 20 min; a wheal diameter of 3 mm greater than that of the negative control was considered as positive.

### 2.3.5. Case history of subjects allergic to horse gram

Case 1: A 49-year-old female (N.T.) had urticaria and wheezing. She avoids eating banana, sapodilla, tomato and citrus fruits. However, SPT was found to be negative to extracts of these fruits. She also strictly avoids horse gram in her diet as she has observed an increase in wheezing within a few minutes after ingestion of any food containing horse gram. She shows moderate SPT for grass pollen mix. The family history for allergy is negative.

Case 2: A 55-year-old male (S.D.) has complaints of urticaria and wheezing as his major health problems. He has observed an increase in wheezing, and also itching in some parts of his body after eating a diet containing horse gram. Currently, he strictly avoids horse gram in his diet. He has also made similar observations with eggplant and was found to be moderately positive to eggplant by SPT. He avoids eating most of the common fruits and raw vegetables; however, he was negative to these extracts by SPT. The family history of allergy is positive.

### 2.3.6. Detection of DBA-specific IgE antibody

#### 2.3.6.1. ELISA for detection of DBA specific IgE antibody

*Dolichos biflorus* agglutinin specific IgE (Sampson, 2001) was determined by ELISA in the sera of atopic subject who had shown positive skin prick test (SPT). Briefly, 15 μg of pure DBA was coated onto wells of 96 well microtiter plate in 50 mM sodium carbonate-bicarbonate buffer of pH 9.6 at $4^0$ C overnight. The plate was thoroughly washed with PBS-T for five times, and then incubated with blocking solution (3% gelatin in PBS) at $37^0$ C for 2 h. The plate was again washed and incubated with normal or allergic subject's sera at 1:3 dilution in PBS-T containing 1% BSA at $4^0$ C overnight. After washing, the plate was incubated with alkaline phosphatase conjugated murine anti-human IgE antibody at 1:1500 dilution. The incubation was carried out at $37^0$C for 2 h. After washing, the plate was developed using *p*-nitrophenyl phosphate as the substrate.

#### 2.3.6.2. Dot-immunoblot of *Dolichos biflorus* lectin

Dot blot (Sampson, 2001) was carried out on nitrocellulose membrane for *Dolichos biflorus* lectin using sera of the normal or DBA sensitized subject as a source of primary antibody. Initially, 15 μg of DBA was applied as a spot on the NC membrane along with con A (a lectin control) and ovalbumin (a non-lectin control). Spots were air-dried and the spotted NC membrane was immersed in a blocking solution (3% gelatin in washing buffer) at 37°C for 2 h. After blocking, membrane was thoroughly washed with wash buffer (PBS-T) for five times and incubated with subject's serum (1:3 diluted with wash buffer containing 1% BSA) at 4°C overnight. The membrane was then washed and incubated with secondary antibody (murine anti-human IgE antibody conjugated with alkaline phosphatase) at 1:1500 dilution in wash buffer containing 1% BSA and 0.5% gelatin at 37°C for 2 h. The membrane was washed and then developed using BCIP-NBT solution at 1:3 dilution. A positive spot appearing blue against a white background indicates the presence of IgE antibody; at this point, the reaction was stopped by washing the membrane with water, dried and stored.

**2.3.7. Isolation of leukocytes containing basophils**

Leukocytes (buffy coat containing basophils) were isolated from 10 mL of heparinized venous blood as described (komiya et al., 2003) using 6% dextran T 700 gradient. The leukocyte layer was washed 4-5 times with isotonic PBS and resuspended in Tris-CAM buffer. The isolated leukocytes were counted using crystal violet; cell viability, as determined by Trypan blue dye exclusion, was 95%.

**2.3.8. Isolation of rat peritoneal exudate cells (PEC)**

PECs were isolated from male Wistar rats (4 weeks-old adults; weight: ~300 g) following the standard procedure (Moreno et al., 2003). Five minutes following the injection of Tyrode buffer, pH 7.4 containing 0.1% BSA, the fluid containing PECs was collected. The residual erythrocytes were removed by treatment with 150 mM $NH_4Cl$ buffer. Next, the cells were pelleted, washed, and resuspended in Tris-CAM buffer. PECs were stained for mast cells using toluidine blue; their viability, as assessed by Trypan blue dye exclusion, was 92%. The PEC preparation was found to contain 15-20% mast cells.

**2.3.9. Histamine release (HR) assay**

After cell count and viability test, nearly 1.5 - 2 x $10^6$ cells /mL were taken for the histamine release assay. This assay consists of three steps (Sampson et al, 1989).

***Step 1: Incubation of cells with lectins to release histamine***

For histamine release, different concentrations of lectin were added (in a volume of 0.25 mL) to polystyrene tubes containing 0.5 mL TrisCAM buffer in an ice bath followed by cells 0.25 mL (from buffy coat and peritoneal exudates) to all tubes (Margo, 1974; Sullivan et al., 1975). The tubes were transferred to a 37°C water bath and incubated for 45 min. Reaction stopped after transferring the tubes to an ice bath. After centrifuging the tubes at 1600 rpm at 4°C for 20 min, the supernatant was poured off and assayed for histamine content (*test release*). In each set of experiment, 10% perchloric acid was added to two tubes (without lectin samples) to a final concentration of 3%; two

additional tubes (without lectin samples) were boiled at 100°C for 10 min; both these manipulations are used to obtain the total histamine content of cells (*complete release).* Blank tubes containing only cells and buffer are used as controls for obtaining the basal level of histamine release (*spontaneous release)*. The histamine release in these tubes was always less than 10% compared to complete release. All the experiments were done in duplicate; there was <2% variation between duplicate tubes.

***Step 2: Manual extraction of released histamine***

The supernatants after centrifugation were poured into separate polystyrene tubes. One mL of this was added to a tube containing 0.3 g NaCl and 1.25 mL butanol. After addition of 0.1 mL of 3 N NaOH, the tubes were shaken and centrifuged at 600 × g at 4°C for 15 min. One ml of butanol (top) layer was removed with a pipette and transferred to another tube containing 0.6 ml of 0.12 N HCl and 1.9 mL of n-heptane. Tubes were shook for 1 min by inversion, and allowed to stand for 5 min for clear separation of two phases. Next 0.5 mL of 0.12 N HCl layer (bottom layer) containing histamine was carefully aspirated using a fine tip and transferred to another tube for histamine assay (Siegel et al., 1990). Care should be taken while removing the HCl bottom layer since butanol may interfere in the histamine assay.

***Step 3: Quantitation of histamine by fluorometric method***

The tubes containing 0.5 mL of the extracted histamine was added with 0.5 mL of 0.12 N HCl, and were transferred to an ice bath (Siegel et al., 1990). To that, 0.2 mL of 1 N NaOH was added, and immediately 0.050 mL of 0.2% *o*-pthalaldehyde (OPT) solution was added and mixed. The reaction was allowed to proceed for 40 min. After incubation, 50 μL of 2 M $H_3PO_4$ was added to stop the condensation reaction and mixed. Then the tubes were kept at room temperature for 15 min. The fluorescence intensity of the solutions was measured using an excitation wavelength of 360 nm and emission wavelength of 450 nm using a spectrofluorometer.

Formulae for the calculation of percent histamine release and percent inhibition

$$\text{Histamine release (A\%)} = \frac{(Pt - Ps)}{(Pc - Ps)} \times 100$$

where, Pt refers to histamine in the test release
Ps refers to histamine in the spontaneous release
Pc refers to histamine in complete release
A% is percent histamine release

$$\text{Inhibition (I \%)} = \frac{1 - (S - B)}{(C - B)} \times 100$$

where, S is percent histamine obtained from test release
C is percent histamine obtained from control release
B is percent histamine obtained from spontaneous release
I % is percent of inhibition for histamine release.

### 2.3.10. Statistical analysis

Each datum represents the mean and standard error of the mean (SEM) of the different experiments under identical conditions. Student's *t*-test was used to make a statistical comparison between the groups.

## 2.4. RESULTS

### 2.4.1. Selection of atopic and non-atopic subjects for the study

Atopic and non-atopic subjects were selected based on detailed case history and clinical symptoms for house dust mite/pollen allergy. Among these groups, the atopic or non-atopic status was confirmed in a representative number of subjects (n = 12 for atopics, and n = 10 for non-atopics) based on the eosinophil count, serum total IgE and plasma/serum histamine levels.

#### 2.4.1.1. Absolute eosinophil counts in non-atopic and atopic subjects

Based on the eosinophil counts and on the incidence of allergic reactions and symptoms, the subjects were classified as non-atopic and atopic subjects. The eosinophil counts were expressed as mean value obtained in each category with standard error of mean. For non-atopics, the value was found to be $302 \pm 11$ with a range of 240 – 350 cells/$\mu$L of blood. In atopic population, the eosinophilic counts were

found to be 776 ± 18 with a range of 680 - 860 cells/μL of blood. The eosinophil counts were increased by ~2.6-fold over the mean value for non-atopic subjects.

**Table 2.1. Absolute eosinophil counts in atopic and non-atopic subjects**

| Subjects | Eosinophil counts/μL* | |
|---|---|---|
| | Mean ± SEM | Range |
| Non-atopic (n=10) | 302 ± 11 | 240 - 350 |
| Atopic (n=12) | 776 ± 18 | 680 - 860 |

*Reference normal value for eosinophil counts = 40–400 cells/μL (Weller et al., 2003); $p \leq 0.001$ ($t = 38.2$)

**2.4.1.2. Total IgE levels in normal and allergic sera**

The total (circulating) IgE levels in the sera of non-atopic and atopic subjects were determined using ELISA. The total IgE exhibited a wide range among atopic subjects (177.3–330.1 IU/mL) as compared to non-atopics range (34.7–43.9 IU/mL).

**Table 2.2. Total IgE levels in atopic and non-atopic subjects**

| Subjects | Total IgE levels | | |
|---|---|---|---|
| | ELISA units (492 nm) | Mean ± SEM | Total IgE* in IU/mL (range) |
| Non-atopic (n=10) | 0.248 – 0.314 | 0.267 ± 0.008 | 34.7 – 43.9 |
| Atopic (n=12) | 0.844 – 1.558 | 1.205 ± 0.120 | 177.3 – 330.1 |

*Reference normal value for serum total IgE = <120 IU/mL (Hamilton and Adkinson Jr, 2003); $p \leq 0.001$ ($t = 13.40$)

The normal range for total IgE is <120 IU/mL. The serum total IgE was found to be significantly higher in atopic subjects, and represents approximately a 5-7 fold increase over the value for non-atopic subjects.

### 2.4.1.3. Serum and plasma histamine levels in allergic and normal subjects

Histamine standard curve was obtained in the range of 0 - 100 ng histamine base (1.66 mg of histamine.2HCl is equivalent to 1 mg histamine base) in a volume range of 0 to 1 mL. A linear response was obtained with a high correlation having correlation coefficient ($R^2$) value of 0.997.

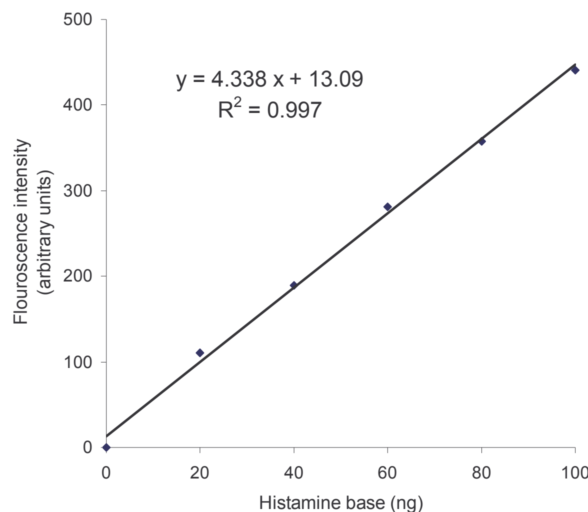



*Fig. 2.1. Histamine standard curve, concentration range 0-20 ng. R2= 0.997*

Serum and plasma histamine levels were quantitated in atopic and non-atopic subjects. The serum and plasma histamine levels were found to be significantly higher in atopic subjects (~6 to 8-fold) as compared to the mean value for non-atopics. The plasma histamine level is 5 to 10-fold lower compared to serum histamine levels in each group. The normal reference value for serum histamine was 5-27 ng/mL where as for plasma histamine was 0.5 to 2 ng/mL in non-atopic healthy subjects.

**Table 2.3. Histamine levels in the serum and plasma of atopic and non-atopic subjects**

| Subject | Histamine content in serum (ng/mL)* | | Histamine content in plasma (ng/mL)# | |
|---|---|---|---|---|
| | Mean ± SEM | Range | Mean ± SEM | Range |
| Non-atopic (n=10) | 28.2 ± 3.6 | 21 - 32 | 1.5 ± 1.2 | 0.6 - 3.0 |
| Atopic (n=12) | 184.2 ± 10.1 | 155 - 215 | 11.6 ± 1.0 | 9.6 – 14.8 |

*Value for non-atopic subjects is 5–27 ng/mL; $p \leq 0.001$ ($t = 15.74$). #Value for non-atopic subjects is 0.5 to 2 ng/mL; $p \leq 0.001$ ($t = 10.64$) (Oguri and Yoneya, 2002).

### 2.4.2. Analyses of DBA for purity

Commercial DBA appears as a single homogeneous band in 12% reducing SDS-PAGE, careful examination revealed two closely spaced bands around 27 kD, in agreement with the literature (Etzler, 1997; 1998). DBA appears as a single peak in RP-HPLC analysis with a retention time of 37.47 min.

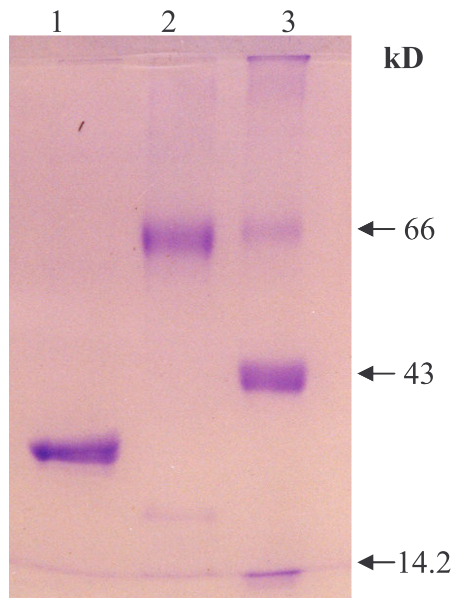



*Fig. 2.2. SDS-PAGE (12%, reducing), Coomassie stained gel: lane 1, DBA; lane 2, BSA; lane 3, (from top to bottom) BSA, OVA, and lysozyme. Protein load: 10 µg.*

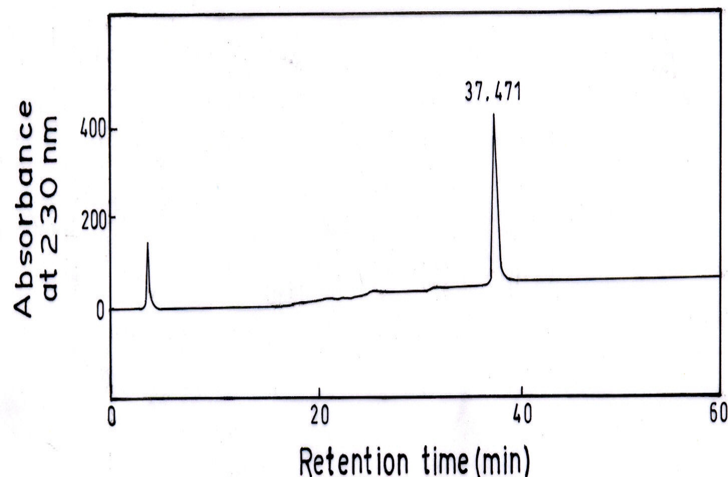



*Fig. 2.3. RP-HPLC, Column: $C_{18}$ (4.5 x 250 mm; particle size 5 µm). Elution: binary gradient of solvent A (0.1% TFA) and solvent B (80% acetonitrile in 0.05% TFA) at a flow rate of 1 mL/min. Protein detection: 230 nm.*

### 2.4.3. Skin prick test, allergen-specific IgE, and dot immunoblot using DBA

Table 2.4 shows the results of SPT with DBA tested on 48 atopic subjects who have generalized symptoms characteristic of allergic conditions (based on case history and SPT), and on 20 non-atopic subjects who do not exhibit any allergic symptoms.

**Table 2.4. Skin prick test of DBA on atopic and non-atopic subjects**

| Subjects | Number of subjects tested | Number of subjects positive | Percent positive | Average wheal/flare diameter (mm) |
|---|---|---|---|---|
| Non-atopic[a] | 20 | 0 | 0.0 | 0.5/0 |
| Atopic[b] | 48 | 10[c] | 20.8 | 3.5/8 |

[a]healthy subjects without any clinical symptoms of allergy (age range: 15-60 y)
[b]subjects displaying characteristic symptoms from any one of the following: asthma, allergic rhinitis, urticaria or food allergy (age range: 15-60 y)
[c]includes 2 subjects sensitized to DBA; individual SPT data are shown in Table 2.6

DBA showed a positive SPT in 10 out of 48 allergic subjects (21%). SPT reactions as assessed by means of wheal/flare diameter were barely positive (designated as +; 3-3.5/5 mm) or moderately positive (designated as 2+; 4-4.5/10-15 mm) compared to the positive control, histamine base (6/25 mm). None of the non-atopic subjects gave a positive SPT (wheal/flare diameter of 0-1/0 mm). The characteristics of the 10 subjects who were positive by SPT to DBA, and their SPT and DBA-specific IgE results are given in Table 2.5.

**Table 2.5. Characteristics of atopic subjects who are positive for Dolichos biflorus agglutinin[a] (DBA) by skin prick test**

| Subjects | Sex/ Age (y) | Allergic history (symptoms)[b] | SPT to DBA (grade)[c] | DBA-Specific IgE ($A_{405}$±SD)[d] |
|---|---|---|---|---|
| YT | M/17 | AR, N, Wh | + | 0.066 ± 0.003 |
| S | F/29 | U | + | 0.078 ± 0.002 |
| NT | F/49 | U, Wh | 2+ | 0.227 ± 0.005 |
| CMN[e] | F/46 | P, U, Wh | + | 0.059 ± 0.001 |
| SD[e] | M/55 | P, U, Wh | 2+ | 0.248 ± 0.003 |
| MG | M/60 | P, U, Wh | + | 0.057 ± 0.001 |
| VK | M/16 | N, Wh | + | 0.089 ± 0.003 |
| SR[e] | F/40 | N, Wh, U | + | 0.045 ± 0.002 |
| BV | F/65 | AR, N, P, Wh | + | 0.063 ± 0.001 |
| GT[e] | F/26 | AR, N Wh, U | + | 0.075 ± 0.002 |

[a]tested at 100 μg/mL concentration in 50% glycerinated-PBS
[b]AR: Allergic rhinitis; N: nasal; P: pharyngeal; U: urticaria; Wh: wheezing
[c]SPT grading based on wheal/flare intensity: +, one-third of histamine control; 2+, two-thirds of histamine control
[d]DBA-specific IgE in the case of non-atopic subjects (n=10) is 0.066
[e]family history of allergy is positive

Only 2 subjects (subjects NT and SD) showed a SPT grading of 2+, and their DBA-specific IgE was 3-fold higher than the value for the other 8 subjects who showed a SPT grading of +. The two DBA-sensitized subjects showed a significant wheal/flare diameter, and the results are shown in the Table 2.5. The case histories of subjects NT and SD are given under 'Materials and Methods'. Serum samples of non-atopic (n = 6) and atopic (n = 6) subjects' were checked for the presence of DBA-specific IgE antibodies. Con A (lectin control) and BSA (non-lectin control) were used as control proteins; their ELISA values for non-atopic and atopic subjects are given as footnotes to Table 2.6.

Table 2.6. Skin-prick test, DBA-specific IgE, total IgE analysis and percent histamine release of non-atopic and atopic subjects

| Samples or subjects | Wheal/flare diameter (mm) | Specific IgE ELISA units[a] ($A_{405}$) | Total IgE ELISA units ($A_{492}$) | Histamine release[b] (%) |
|---|---|---|---|---|
| Negative control | 0-1/0 | n.a.[c] | n.a. | n.a. |
| Positive control | 6.0/20 | n.a. | n.a. | n.a. |
| Case 1 (N.T.) | 4.0/10 | 0.227 | 0.844 | 68 |
| Case 2 (S.D.) | 4.5/15 | 0.248 | 1.181 | 71 |
| Non-atopic subjects[d] | 0.5/0 | 0.066 | 0.267 | 25 $\pm$ 1.3 |
| Atopic subjects[d] | 3.5/5 | 0.083 | 0.933 | 28 $\pm$ 1.1 |

[a]Value for non-lectin control (BSA) (n=6): 0.018 (non-atopic); 0.020 (atopic);
value for lectin control (Con A) (n=6): 0.056 (non-atopic); 0.099 (atopic)
[b]measured at 2 $\mu$g/mL DBA concentration
[c]n.a. = not applicable
[d]mean value of different parameters for these subjects are shown (n = 10)

The ELISA value for DBA-specific IgE for atopic subjects was very similar to that seen for non-atopic subjects, although the total IgE level of atopic subjects is approximately 3.5-fold higher than that of non-atopic subjects (Table 2.6). However,

the DBA-sensitized subjects NT and SD, who were found to have a positive case history for the ingestion of horse gram, showed significantly higher ELISA values (0.227 for NT, and 0.248 for SD), for DBA-specific IgE (Tables 2.5 and 2.6). These two subjects also showed an increase of 3.2-fold and 4.5-fold, respectively, in the values for total IgE compared to non-atopic subjects.

In the case of the DBA-sensitized subjects NT and SD, IgE-dot immunoblot for DBA was found to be positive to DBA and negative to OVA and con A which were used as control proteins (Fig. 2.4). The dot immunoblot was, as expected, negative for the serum of non-atopic subject (N). This clearly reveals the presence of DBA-specific IgE in two cases that have positive case history (to the ingestion of horse gram), and were positive by SPT and allergen-specific IgE. However, these two subjects were unavailable for follow-up by double-blind placebo-controlled food challenge.

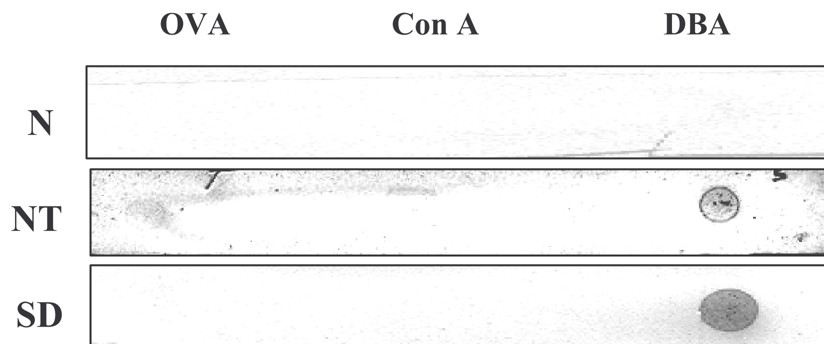



*Fig. 2.4. Dot immunoblot of DBA using non-atopic and atopic subjects' sera (subjects positive to DBA by skin-prick test). N: Non-atopic subject; NT: DBA-sensitized atopic subject NT (positive to DBA by SPT); SD: DBA-sensitized atopic subject SD (positive to DBA by SPT).*

### 2.4.4. Histamine release (HR) from DBA-sensitized subjects

The leukocytes containing basophils were obtained from the heparinized venous blood of DBA-sensitized subjects (subjects NT and SD) for performing HR assay. The HR was found to be maximum at 2 μg/mL DBA (Fig. 2.5); the values were found to be 68% for subject NT, and 71% for subject SD. There is approximately a 3-fold increase in HR compared to a non-atopic subject who showed 25% HR under identical conditions.

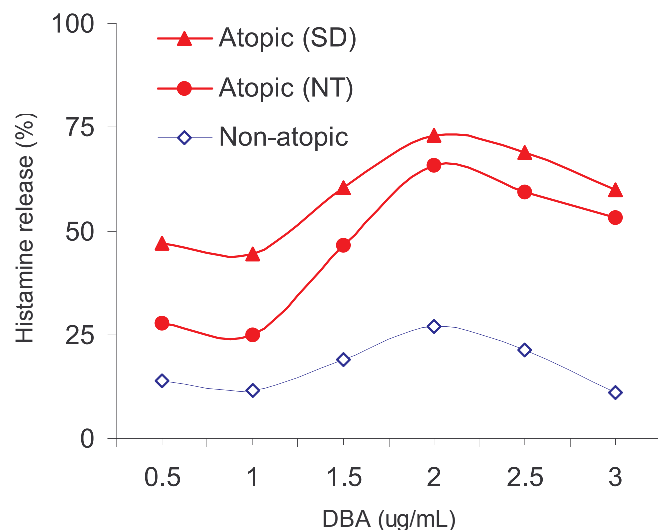



*Fig. 2.5. Histamine release from the leukocytes of two DBA-sensitized subjects (subjects NT and SD) and non-atopic subject, as a function of DBA concentration. Leukocyte concentration: $2 \times 10^6$ cells/mL. Following extraction and derivatization with OPT, the released histamine was determined by fluorometry. Details are given under 'Materials and methods'.*

### 2.4.5. HR from non-atopic and atopic subjects using DBA

In order to find out the DBA concentration, which gives maximum HR in the case of atopic subjects (n = 3), the HR assay was performed in the concentration range of 0.0001-10 µg/mL. The results are shown in Fig. 2.6.

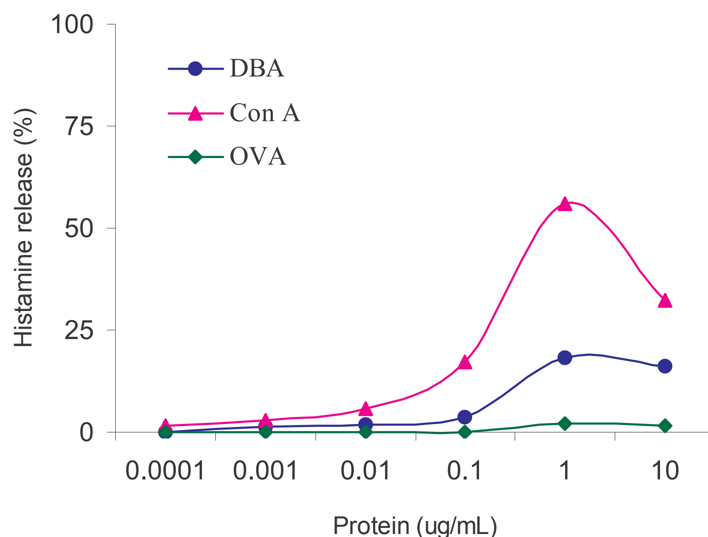



*Fig. 2.6. Histamine release from the leukocytes of human atopic subjects (n = 3) as a function of DBA concentration in the range of 0.0001 to 10 $\mu$g/mL. Con A and OVA were used as lectin and non-lectin controls, respectively.*

The HR from non-atopic and atopic subjects as a function of DBA, con A, and OVA in the narrow concentration range of 0.5-3 μg/mL is shown in Fig. 2.7. Maximum HR was seen at 2 μg/mL in the case of DBA and con A. DBA shows a HR of ~25% and 28% in non-atopic (n = 4) and atopic (n = 5) subjects, respectively, indicating that there is no significant difference in HR from these two groups. Con A shows a typical bell-shaped curve for HR with a maximum at 2 μg/mL. The HR was ~40% in non-atopic subjects and ~73% in atopic subjects. OVA (negative control) show a HR of only 4% in the case of non-atopic subjects and 6% in the case of atopic subjects. Other non-lectin proteins (BSA, lysozyme) showed even lesser HR than OVA (data not shown).

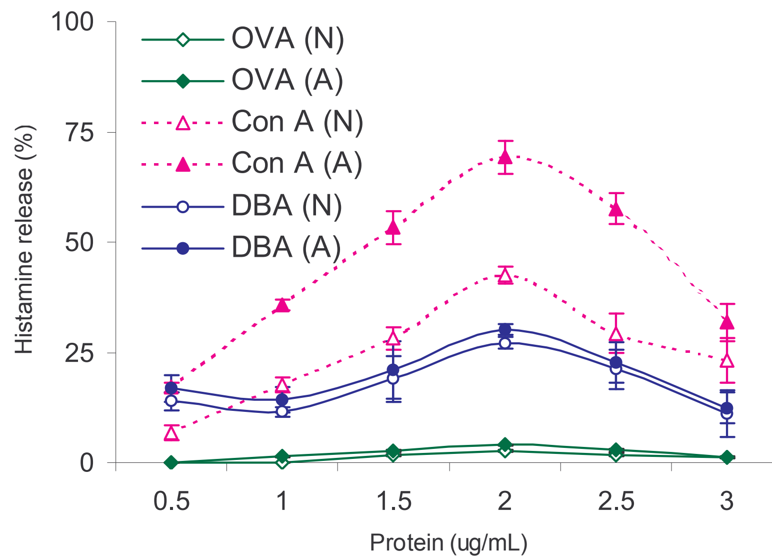



*Fig. 2.7. Histamine release from the leukocytes of human non-atopic and atopic subjects as a function of protein concentration (OVA, non-lectin control; Con A, lectin control; DBA, Dolichos biflorus agglutinin). (N): non-atopic subjects (n = 4; open symbols); (A): atopic subjects (n = 5; closed symbols).*

### 2.4.6. Inhibition of DBA-induced HR by sugars

The effect of various sugars on DBA-induced HR (22%) at 2 μg/mL is shown in Fig. 2.8. D-Mannose, D-galactose, and GlcNAc (all at 100 μg/mL) do not cause any inhibition of HR. However, GalNAc at 50 μg/mL was found to inhibit the HR by ~4-fold (6% HR); this effect was more pronounced at 100 μg/mL (2% HR). In the absence of $Ca^{2+}/Mg^{2+}$ from Tris-CAM buffer, the HR was very low, and is comparable to the release by a non-lectin protein (BSA).

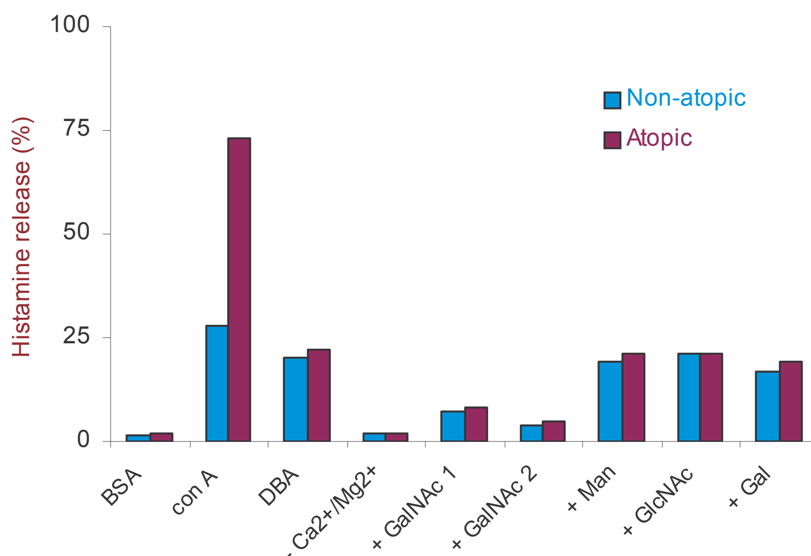



*Fig. 2.8. Histamine release and its inhibition by various sugars. HR from atopic (n = 2) and non-atopic (n = 2) leukocytes ($2\times10^6$ cells/mL) using DBA at 2 μg/mL concentration. Con A and BSA are lectin and non-lectin controls, respectively. The concentration of sugars used for inhibition of HR induced by DBA is 100 μg/mL, except GalNAc 1 (50 μg/mL). HR by DBA in the absence of $Ca^{2+}/Mg^{2+}$ (Tris-CAM buffer without metal ions) is also shown.*

### 2.4.7. HR from rat peritoneal exudates cells using DBA

The HR from rat PECs using DBA in the concentration range of 1-5 μg/mL is shown in Fig. 2.9. The release was found to be maximum (48%) at 4 μg/mL. Con A shows the characteristic bell-shaped curve with a maximum HR of 57% at 3 μg/mL, whereas OVA showed a maximum release of only 9% at 4 μg/mL.

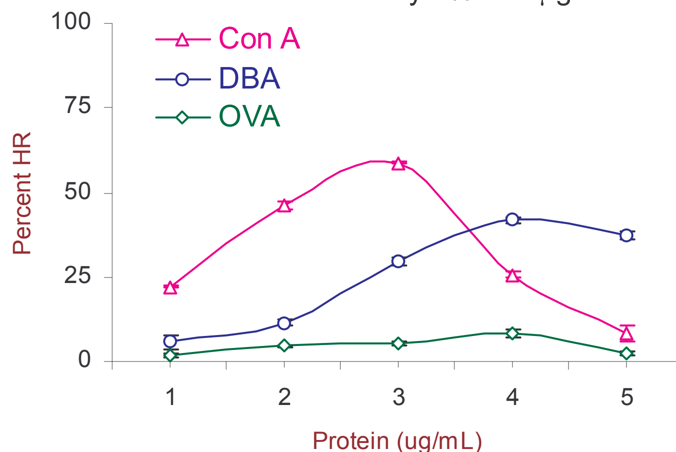



*Fig.2.9. HR from rat peritoneal exudate cells as a function of protein concentration. PEC concentration: $2\times10^6$ cells/mL. The results represent mean of three analyses. Released histamine was determined by fluorometry as given under 'Materials and Methods'.*

## 2.5. DISCUSSION

Horse gram, consumed widely in India, is a valuable protein-supplement containing 22% protein (CSIR, 1952). The lectin is one of the major proteins as is the case with many legume lectins (Van Damme et al., 1998; Etzler, 1997; 1998). The present study deals with the effect of horse gram lectin on mast cells/basophils of non-atopic and atopic subjects, and to investigate whether a common dietary lectin (DBA) mediates non-allergic food hypersensitivity.

SPT of DBA revealed that approximately one-fifth of the atopic subjects showed a positive reaction, whereas there was no reaction in the case of non-atopic subjects. A positive SPT of 21% to a purified protein (DBA) from horse gram appears to be unusual for food allergy, since the incidence of food allergy in adults is generally 2-4% (Sampson, 2001). However, considering that 20-25% of the general population is atopic (ISAAC, 1998), the SPT results for DBA translates to 4-5% of the general population. This may be due to the non-specific interaction of DBA with the mast cells *in vivo.* It has been shown by Hormia *et al*., (1988) that DBA reacts selectively with mast cells in human connective tissue cells and epithelial cells. In the case of 10 atopic subjects who showed a positive SPT for DBA, the total IgE level was, in general, 3-5 folds higher than in non-atopic subjects. Despite this increased total IgE level, the magnitude of HR from atopic subjects is similar to that seen from non-atopic subjects.

The DBA-specific IgE was found to be absent in atopic subjects who were positive for DBA by SPT (with the exception of 2 subjects). The two subjects (subjects NT and SD) who are allergic to horse gram by case history, were identified as sensitized to DBA as confirmed by the presence of DBA-specific IgE by ELISA and dot immunoblot. While the total IgE levels of these two subjects were similar to those of other atopic subjects (~4-fold higher compared to non-atopic subjects), DBA-specific IgE is about 3-fold higher in these two subjects as compared to atopic subjects. These results correlate very well with the case history of the subjects wherein they have described the allergic symptoms experienced following ingestion of foods prepared with horse gram. The *in vitro* basophil activation using the leukocytes of DBA-sensitized subjects show a

significant increase in HR upon incubation with DBA indicating the degranulation of basophils *via* allergen-IgE (binding site) interactions.

Allergy to ingestion of horse gram has not been reported so far. The description of the two subjects in this study appears to indicate the first few cases of food allergy to horse gram; DBA has been identified as the allergen, and has been named Dol b Agglutinin. Lectins have been reported earlier as minor allergens (Burks et al., 1994; Baur et al., 1996; Weichel et al., 2006; Jenkins et al., 2005) in only three plant foods, namely, peanut (Ara h Agglutinin) (Burks et al., 1994), soybean (Gly m Lectin) (Baur et al., 1996), and wheat (Tri a 18; wheat germ agglutinin or WGA) (Weichel et al., 2006). Lectins have been ranked 9$^{th}$ in their assignment as plant food allergen families in Pfam database (Jenkins et al., 2005), and are considered as minor allergens.

HR from the leukocytes of non-atopic and atopic subjects in the presence of DBA are similar in pattern. This clearly indicates that DBA does not depend on the basophil IgE density to interact and cause activation to release biological mediators. Since mast cell-bound IgE is a glycoprotein rich in complex type glycans (~12%), the composition of the *N*-linked glycans on its ε-chains was examined. The glycans comprise of core and non-core GlcNAc, mannose, fucose, galactose, and sialic acid; notably, GalNAc was not present (Arnold et al., 2004; Hajdukovic-Dragojlovc et al., 1997). The lower magnitude of HR (20-25%) by DBA may be due to its interaction with some other glycoproteins/glycolipids containing terminal GalNAc residues on the surface of basophils; the low level of HR induced by DBA can be inhibited using the specific saccharide, GalNAc. This is in contrast to con A wherein the HR is higher in non-atopic subjects, and comparatively more so in atopic subjects. Con A-induced HR is dependent on the density of IgE present on basophils (Magro, 1974; Siraganian and Siraganian, 1974; 1975; Helm and Froese, 1981). Con A cross-links the glycans of two adjacent IgE which provides the basis for degranulation, and the effects are identical to that of allergen-mediated hypersensitivity reactions (Schwartz, 2004; Galli et al., 2005).

DBA was found to release histamine from rat peritoneal mast cells similar to, but not to the same extent as, con A and KM+, a mannose-binding lectin from the nutritious

seeds of jackfruit (*Artocarpus integrifolia*) (Moreno et al., 2003). Roberts *et al*. (1990), found that dermal and subepidermal mast cells in the rat and mouse, and both mucosal as well as dermal human mast cells showed very similar lectin-binding properties to each other. It may be speculated here that the HR from rat PECs and positive SPT to DBA in 21% of atopic subjects may be due to the binding of DBA to similar GalNAc-containing glycoproteins on mast cells. Alternatively, the glycosylation pattern of mast cells in a sub-population of atopic subjects may be different in terms of *O*-glycosylation. In eukaryotes, GalNAc has been described as a glycan component in *O*-linked glycoproteins such as mucin, fetuin, human gonadotrophins, antifreeze glycoproteins, and Tamm-Horsfall mucoprotein (Spiro, 2002).

### 2.6. SUMMSRY AND CONCLUSION

Horse gram (*Dolichos biflorus*) is widely consumed in the tropical south Asian countries including rural areas of India. Since *Dolichos biflorus* agglutinin (DBA) is an important dietary lectin in horse gram, we have studied its effect on the degranulation of mast cells and basophils of atopic subjects. Among the atopic group, 10 of 48 subjects (21%) were found to be positive by SPT to DBA, and none were positive in the non-atopic group (n=20). Two subjects out of the ten who tested positive for DBA by SPT were found to be sensitized to DBA as revealed by the presence of specific IgE by ELISA and dot-blot. The HR was found to be 2-3 folds higher in DBA-allergic subjects than in non-atopic and atopic subjects. Basophil HR by DBA was found to be similar in both non-atopic and atopic subjects. However, DBA induces activation of mast cells *in vivo* in a sub-population (21%) of atopic subjects. Two subjects have been identified as having food allergy to horse gram based on the presence of DBA-specific IgE with a positive correlation to basophil HR. This is the first report of food allergy to horse gram, and DBA has been identified as an allergen. The DBA has now been designated as Dol b from the allergome database.

# Chapter 3

# Purification of potato lectin utilizing pentose colorimetric assay and development of a glycoprotein binding assay

## 3.1. INTRODUCTION

Potato (*Solanum tuberosum*, Solanaceae), is the only major tuber crop that is grown in temperate regions. It is also the most important tuber crop in terms of production accounting for about 45% of the total world production of all tuber crops (Shewry, 2003). The spread of potato from its center of origin in the high Andes of South America to other parts of the globe, and the historical consequences of this have been well documented (Messer, 2000). Potato tubers are derived from swollen stems, although they are generally subterranean. The major protein in potato tuber is the storage protein, patatin (Osborne and Campbell, 1896). Although the major biochemical component in potato is starch, it contains 1.6 g protein per 100 g tuber.

Hydroxyproline-rich glycoproteins (HRGPs) of higher plants occur predominantly at the cell surface as covalent wall networks (extensins) or as soluble mucin-like molecules (gums and arabinogalactan-proteins) (Kieliszewski and Lamport, 1994). Additionally, there is an expanding inventory of intra- and extra cellular chimeric proteins containing an HRGP-like domain or molecule. This group includes the solanaceous lectins from potato, tomato (*Lycopersicon esculentum*) and thorn-apple (*Datura stramonium*) (Allen et al., 1978; Desai et al., 1981; Kilpatrick, 1983; Merkle and Cummings, 1987; Miller et al., 1992). The best characterized of these HRGP chimeras is potato lectin, a developmentally regulated, chitin-binding glycoprotein (50% carbohydrate, w/w).

Potato lectin, also known as *Solanum tuberosum* agglutinin (STA) (Kieliszewski et al., 1994), is a blood group-nonspecific lectin (pan-agglutinin) present in potato tubers, and is specific for chitin (poly *N*-acetyl-D-glucosamine) (Allen et al., 1996; Van Damme et al., 2002); the highest specificity is for trimer and tetramer of GlcNAc. The lectin also binds poly-*N*-acetyllactosamine structures (Van Damme et al., 2002). It is an unusual glycoprotein with high contents of half-cystine and hydroxyproline residues; almost all hydroxyproline residues are substituted with oligo-L-arabinose (mostly trimers and tetramers in the furanose form with $\beta$-linkage) and a few serine residues are substituted

with D-galactose (Spiro, 2002). Potato lectin is dimeric comprising of two identical monomers of ~55 kD, fifty percent of its mass being carbohydrate.

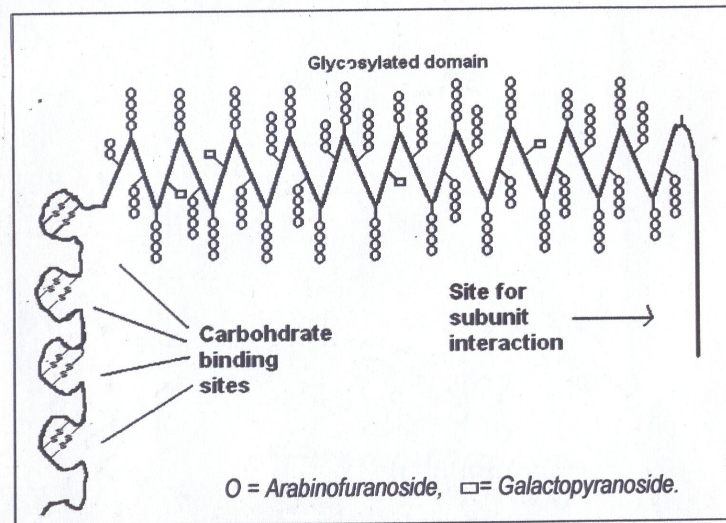



***Fig. 3.1.*** *Hypothetical model of the structure of potato lectin (Allen, 1983). It is a arabinose rich glycoprotein with 50% of carbohydrate by weight.*

Potato lectin consists of at least two evolutionary autonomous domains: a lectin module fused to an HRGP module (Allen et al., 1978; Pont-Lezica et al., 1991). The lectin module possesses a β-turn secondary structure (Matsumoto et al., 1983; Van Holst et al., 1986) and has a composition reminiscent of other plant chitin-binding proteins in that it is rich in cystine and glycine, contains tyrosine and tryptophan, and has no glycans (Nagata and Burger, 1974; Allen et al., 1978; Chrispeels and Raikhel, 1991). In contrast to the lectin domain, the HRGP domain of potato lectin is rich in hydroxyproline, is highly glycosylated with abundant arabinose and minor amounts of galactose (Allen et al., 1978), and has a polyproline-II secondary conformation (Van Holst et al., 1986). The Hyp-rich domain compositionally resembles members of the extensin family and has Ser-$Hyp_4$ repetitive pentamers containing arabinosyloligosaccharides *O*-linked to Hyp, and monogalactosylserine (Kieliszewski and Lamport, 1994).

The method of Allen and Neuberger (1973) is widely used for the purification of STA. Following ammonium sulfate precipitation step, this method involves 5

chromatographic steps in the order: DEAE-cellulose, CM-cellulose, Sephadex G-100 (twice), and SP-Sephadex. Apart from this, other methods of purification have been described: affinity chromatography using N, N', N"-triacetyl chitotriose-Sepharose matrix (Desai and Allen, 1979) or fetuin-Sepharose matrix (Owens and Northcote, 1980), and chromatofocusing involving two steps (McCurrach and Kilpatrick, 1986). Although affinity chromatography and chromatofocusing techniques are very powerful, they require specialized and expensive reagents. During the course of this investigation on the modulatory effects of purified potato lectin on the cells of the immune system, the necessity for a simpler and shorter procedure for purifying potato lectin was felt compared to the five lengthy chromatographic steps described in the classical procedure (Allen and Neuberger, 1973). Here, the purification of potato lectin using only two chromatographic steps beyond the ammonium sulfate precipitation step of Allen and Neuberger (Allen and Neuberger, 1973) is described.

Since STA is very rich in arabinose (92% carbohydrate) (Allen and Neuberger, 1973; Allen et al., 1996; Van Damme et al., 2002), the detection of potato lectin by using a colorimetric assay for pentoses (Bial's test) (Ashwell, 1957; Dische, 1962; Chaplin, 1986), which involves the use of ferric chloride-orcinol (FCO) reagent was attempted. Although hexoses do react under the conditions of the assay, they give only a faint, yellow color that is masked by the blue-green color given by pentoses (Ashwell 1957; Dische, 1962). The pentose assay has been used earlier to quantitate free pentoses like xylose, ribose, or arabinose in biological samples (Ashwell 1957; Dische, 1962), and for the specific determination of RNA (Almog and Shirey, 1978). However, the FCO assay has, so far, not been used for the detection of glycoproteins containing pentoses. In this paper, the use of pentose assay has been incorporated to develop a modified purification procedure for detecting STA in each step, and its advantages are described.

Although potato lectin has optimal specificity for chitotrimer and chitotetramer (Van Damme et al., 2004), it appears that it may also bind to core chitobiose units found in *N*-linked glycoproteins. Since dietary lectins are an important constituent of dietary proteins in many plant foods (Puztai et al., 1990), and are known to bind to the lining of

gut wall and make it more leaky, so that more undigested lectins can enter into the systemic circulation (Kjaer and Frokiaer. 2005). By that they can interact with specific sugars present on the N-glycans of various glycoproteins to bring out many biochemical and pharmaceutical functions. Most lectins were known to have resistance to heat and gut proteases, and are found to retain their biological activity even after cooking, In the present study an attempt was made for potato lectin to assess its stability after heat processing and glycoprotein binding ability which will help in providing biological significance in the *in vivo* system.

## 3.2. MATERIALS

Potato tubers were procured from the local grocery. Sephadex G-75 (fractionation range 10-50 kD), SP-Sepharose (fast flow) cation-exchanger (wet bead size: 45-165 μ), gum arabic (Acacia gum), avidin, soybean agglutinin, tomato lectin (*Lycopersicon esculentum* agglutinin), and *Dolichos biflorus* agglutinin were products of Sigma-Aldrich Co., St. Louis, MO, USA. Ovalbumin, bovine serum albumin (BSA), lysozyme, concanavalin A and horseradish peroxidase (HRP) were purchased from Bangalore Genei, Bangalore, India. Patatin (major storage protein of potato) obtained during the purification of potato lectin in this study was used. Flat-bottom 96-well microtiter plates (MICROLON) were bought from Greiner Bio-One GmbH, Frickenhausen, Germany. All other chemicals and reagents used in this study were of analytical grade.

## 3.3. METHODS

### 3.3.1. Purification of potato lectin from potato tubers

#### 3.3.1.1. Preparation of Potato extract

Potato tubers (100 g) were peeled washed, cut into pieces and homogenized by grating, and transferred to 2 volumes of 0.1 M sodium acetate buffer, pH 3.8, containing 2 mM sodium metabisulfite as an antioxidant to prevent browning. After keeping at 4°C for 2 h, the extract was filtered through a muslin cloth, and the filtrate was centrifuged at

5000 x *g* at 4°C for 15 min. The clear pale yellowish supernatant obtained was stored at 4 ºC; the precipitate was resuspended again in the same buffer and the procedure repeated twice.

**3.3.1.2. Ammonium sulfate precipitation and ultra-filtration**

The supernatant was subjected to sequential 20% and 60% ammonium sulfate saturation as described in the method of Allen and Neuberger (1973). Ammonium sulfate $(NH_4)_2SO_4$ (10.6 g/L) was added to the combined supernatants to produce 20% saturation at 4 ºC. The precipitate was collected by centrifugation at 9000 x g for 20 min and discarded; next, $(NH_4)_2SO_4$ (17.5 g/L) was added to the supernatant to produce 60% saturation at 4 ºC, the precipitate was left to settle overnight and then collected by centrifugation at 9000 x g for 20 min. The precipitate was resuspended in distilled water, dialyzed extensively against water, and concentrated by ultrafiltration using Amicon stirred-cell fitted with Millipore YM-10 membrane (mol. wt. cut-off = 10 kD) in the cold. After concentration, the filtrate was discarded and the retentate was re-centrifuged at 9000 x g for 20 min. The supernatant (10 mL) obtained was further concentrated to 3 mL by lyophilization for Sephadex G-75 column chromatography.

**3.3.1.3. Gel permeation chromatography using Sephadex G-75**

The concentrated material obtained from ultrafiltration (3 mL) was subjected to gel filtration on Sephadex G-75 (2.5 x 110 cm) column chromatography, which was pre-equilibrated with Tris-buffered saline (TBS). Elution was carried out at a flow rate of 10 mL/h using TBS, and the fraction volume was 3 mL. The active fractions containing pentose-positive material were pooled (component I), and dialyzed against 50 mM sodium acetate buffer, pH 4.0 for subjecting to cation exchange chromatography.

**3.3.1.4. Cation-exchange chromatography on SP-Sepharose FF**

Component I obtained from the Sephadex G-75 was applied on to the cation-exchanger SP-Sepharose FF (1 x 13 cm; bed volume, 10 mL) column, which had been

pre-equilibrated in 50 mM sodium acetate buffer, pH 4.0. The flow-through was collected, and the column was washed with 5 bed volumes of the same buffer to remove non-specifically bound protein components. Bound proteins were eluted by step-wise elution with different NaCl concentrations (steps of 0.1, 0.25, 0.5, 0.75, 1, and 1.5 M) in the starting buffer. Volume of buffer used for each step was 30 mL, the flow rate was maintained at 25 mL/h and the fraction volume was 5 mL.

### 3.3.2. Ferric-orcinol assay (Bial's test)

This is a colorimetric assay for the detection of free pentoses in biological samples (Chaplin, 1986). In this assay, 200 µL of the sample was taken, then 200 µL of reagent A (10% w/v TCA) was added, the samples were heated at 100°C for 15 min. The tubes were cooled rapidly at 25°C and 1.2 mL of reagent B (1.15% w/v ferric ammonium sulfate and 0.2% w/v orcinol in 9.6 M HCl) was added and mixed well. The samples were again heated at 100°C for 20 min, and then cooled to room temperature. The absorbance of the blue-green color was measured at 660 nm. The calibration curve for D-arabinose was prepared in the 0-40 µg range (volume range: 0-200 µL). Glycoproteins such as patatin, ovalbumin, horseradish peroxidase, *Dolichos biflorus* agglutinin, and soybean agglutinin were used as control glycoproteins devoid of pentoses. Non-glycoproteins such as BSA, lysozyme, and concanavalin A were used as control proteins in this assay.

### 3.3.3. Hemagglutination (HA) activity and sugar inhibition assays

#### 3.3.3.1. Preparation of 2% erythrocyte suspension

Five milliliters of fresh rabbit (or human) blood was collected and put into 5 mL of Alsever's solution (20.59 g glucose, 8 g sodium citrate, 4.29 g NaCl and o.55 g citric acid dissolved and made up to 200 mL distilled water; working dilution was 1:5), swirled and mixed thoroughly. After centrifugation at 1000 x g in the cold for 10 min, the supernatant was removed and the pelleted RBC was washed 3-4 times with saline (0.9% NaCl), and finally resuspended in PBS, pH 7.4. After adjusting the RBC suspension to 2% (using

Klett photometer with 660 nm filter; reading of 400 represents 2% RBC concentration), 1% crude trypsin was added and incubated at 37°C for 1 h. After incubation, the cells were centrifuged at 1000 x g for 10 min at 4°C, washed with saline and readjusted to 2% concentration for use in the HA assay.

#### 3.3.3.2. HA activity and sugar inhibition assays

HA activity of the purified lectin and the sugar inhibition assays were carried out using trypsinized rabbit or human erythrocyte suspension as described by Burger (Burger, 1974). Briefly, a 2% suspension of rabbit or human erythrocytes (0.2 mL) were added to a serially diluted lectin solution, gently mixed and incubated at $37^0$C for 1 h and the agglutination was visualized. The amount of protein present at the highest dilution represents the minimum quantity of protein necessary for agglutination and is taken as the titer. One unit of HA activity is the concentration of the protein at the titer. The specific agglutination activity is given as the number of HA units per mg of protein.

For sugar inhibition, different concentrations of the sugar solutions (in a volume of 0.1 mL) in each of the wells were preincubated with 0.1 mL lectin solution at $37^0$C for 1 h before the addition of 0.2 mL 2% trypsinized erythrocytes. Agglutination was visualized; the extent of inhibition by different sugars or glycoproteins was assessed by their ability to inhibit the hemagglutination activity of lectin (Burger, 1974).

### 3.3.4. SDS-PAGE and protein assay

Selected pools from the chromatographic steps were analyzed by 12% SDS-PAGE (reducing), as per the procedure of Laemmli (1970). The protein bands were visualized either by coomassie blue or silver staining. Protein assay was carried out as per the procedure of Bradford (1976), using BSA as the standard.

### 3.3.5. Periodic acid-Schiff (PAS) staining

PAS staining for glycoprotein (Zacharius, 1969) was used to confirm the glycoprotein nature of purified potato lectin. Purified potato lectin and potato extracts at

various stages of purification, were run on 10% SDS-PAGE under reducing conditions; following fixation in 12.5% TCA solution for 1 h, the gel was incubated in the dark with 1% periodic acid solution for 1 h. The gel was thoroughly washed with water, and the bands were developed by incubation with Schiff reagent (Schiff's fuchsin-sulfite reagent) at $4^0$C for 1 h in the dark. After the development of colored bands, the reaction was stopped by transferring the gel into a solution of 7% glacial acetic acid.

### 3.3.6. Reverse-phase HPLC

Reverse-phase HPLC analysis was carried out using a $C_{18}$ column (4.5 x 250 mm; particle size 5 µm) in a Shimadzu LC-10A HPLC system (Shimadzu Corp., Kyoto, Japan). Elution was carried out using a binary gradient of solvent A (0.1% TFA) and B (70% acetonitrile in 0.05% TFA) at a flow rate of 0.8 mL/min. Protein detection was monitored at 230 nm.

### 3.3.7. Preparation of chitosan oligomers

Chitosan oligomers were obtained by digestion of 500 mg chitosan with 5 mg pepsin at pH 5, 45°C for 6 h, followed by neutralization, centrifugation, and lyophilization of the supernatant (Vishukumar and Tharanathan, 2004). The composition of chitosan oligomers by HPLC analysis (aminopropyl column) was found to be 78.2% chitotetraose, 1.6% chitotriose, 0.9% chitobiose, 18.2% GlcNAc, and 1.1% GlcN (D-glucosamine).

### 3.3.8. Glycoprotein-binding assay

Microtiter wells were coated with 30 µg of protein (100 µL volume) in 0.1 M carbonate-bicarbonate buffer, pH 9.6 at 4°C overnight. After blocking the wells using 3% gelatin in PBS and subsequent washings, the microtiter plate was incubated with 0.1 mg/mL horseradish peroxidase (100 µL) or avidin-alkaline phosphatase (1:2000 dilution; 100 µL) at 37°C for 2 h. Color development was carried out using *o*-phenylenediamine/$H_2O_2$ substrate system and absorbance was read at 492 nm for HRP

and for avidin-AP *P*-nitro phenyl phosphate was used as substrate system and absorbance was read at 405 nm.

### 3.3.9. Preparation of raw and heat-processed potato extracts

For raw potato extract (RPE), 50 g of peeled potato tubers were taken and blended with 50 mL of sodium acetate buffer of pH 4.0. The extract was passed through a muslin cloth and then filtered using Whatman no. 1 filter paper. The extract was then centrifuged in the cold at 5200 x g for 15 min.

For heat-processed potato extract (HPPE), 50 g of peeled potato tubers were taken and suspended in 50 mL of sodium acetate buffer pH 4.0. The contents were boiled over a hot plate for 20 min. After cooling to room temperature, 1 mL of the supernatant was taken for analysis (HPPE supernatant), and later, the remaining contents were blended at 25°C. The extract was passed through a muslin cloth and then filtered using Whatman no. 1 filter paper. The extract was then centrifuged in the cold at 5200 x g rpm for 15 min.

## 3.4. RESULTS

### 3.4.1. Preparation of raw potato extract from potato tubers

Immediately after peeling the skin, potato tubers were cut into pieces and soaked in extraction buffer (sodium acetate buffer, pH 3.8 containing 2 mM sodium metabisulfite as an antioxidant to avoid browning reaction). The extract was clear and pale yellowish in color; it was subjected to sequential 20% and 60% $(NH_4)_2SO_4$ precipitation, followed by ultra filtration and extensive dialysis (cut off 10 kD). At this stage, the extract was light brown in color, and its pH was 7.2. Raw potato extract was concentrated to dryness by lyophilization.

### 3.4.2. Potato lectin purification from potato tubers

Potato lectin was purified to homogeneity by two chromatographic steps; gel permeation and cation exchange, following ammonium sulfate precipitation.

#### 3.4.2.1. Gel permeation chromatography on Sephadex G-75

The resolubilized precipitate obtained from 60% ammonium sulfate saturation of potato extract was concentrated by ultrafiltration using YM-10 membrane, and gel filtered on Sephadex G-75. The elution profile (Fig. 3.2) shows the resolution of proteins in potato extract (of molecular mass >10 kD) into three components. Among the three components seen, only fractions representing component I were positive in the FCO assay; HA activity of component I was found to be ~165 units/mg protein. Pooled component I containing functionally active STA was subjected to SDS-PAGE (reducing), and the gel pattern shows the presence of three bands, all having molecular mass >40 kD (Fig. 3.4, lane 4). The major intense band at ~45 kD represents the abundant storage protein of potato, namely, patatin (Shewry, 2003). Component II which was found to be negative in both the FCO and HA assays, contained several proteins all having a molecular mass of <35 kD (Fig. 3.4, lane 5).

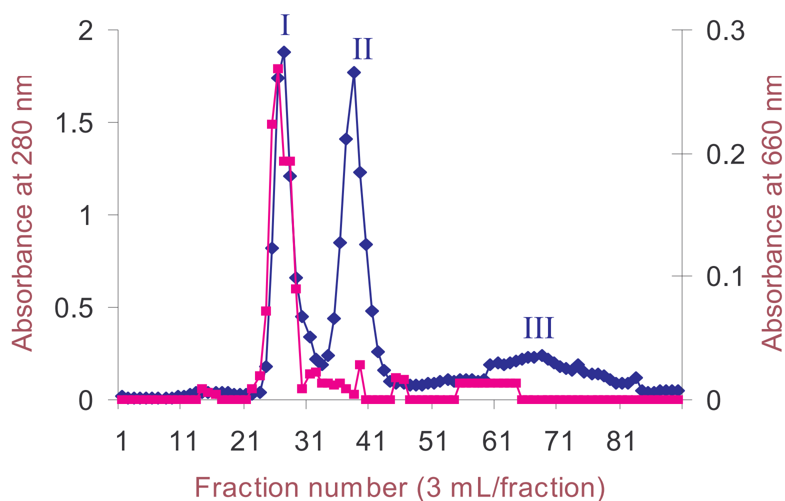



***Fig. 3.2.*** *Gel filtration of concentrated resolubilized precipitate from 60% ammonium sulfate saturation of crude potato extract on Sephadex G-75 (2.5 x 110 cm) using 10 mM Tris-HCl, pH 7.4, containing 140 mM NaCl as eluant at 25°C. Flow rate: 15 mL/h. Protein detection: absorbance at 280 nm (-♦-). Pentose detection (FCO assay): absorbance at 660 nm (-■-).*

#### 3.4.2.2. Cation exchange chromatography on SP-Sepharose FF

Chromatography of pooled component I was carried out on SP-Sepharose cation-exchanger at pH 4. The elution profile obtained using step-wise NaCl elution,

was monitored by absorbance at 280 nm, FCO assay and is shown in Fig. 3.3. Based on FCO assay, almost all the fractions eluting at 100 mM NaCl concentration (component Ia in step elution A, Fig. 3.3) were found to be pentose-positive. The HA activity was remarkably higher compared to the 60% ammonium sulfate precipitate of potato extract, and also component I of Sephadex G-75. The HA activity was found to be ~3891 units/mg protein. Component Ib obtained upon elution with 0.25 M NaCl (step elution B, Fig. 3.3) was found to have a higher amount of protein than component Ia. Only a small amount of protein was found to elute in component Ic (step elution C using 0.5 M NaCl, Fig. 3.2). Both components Ib and Ic were found to be negative in the FCO and hemagglutination assays.

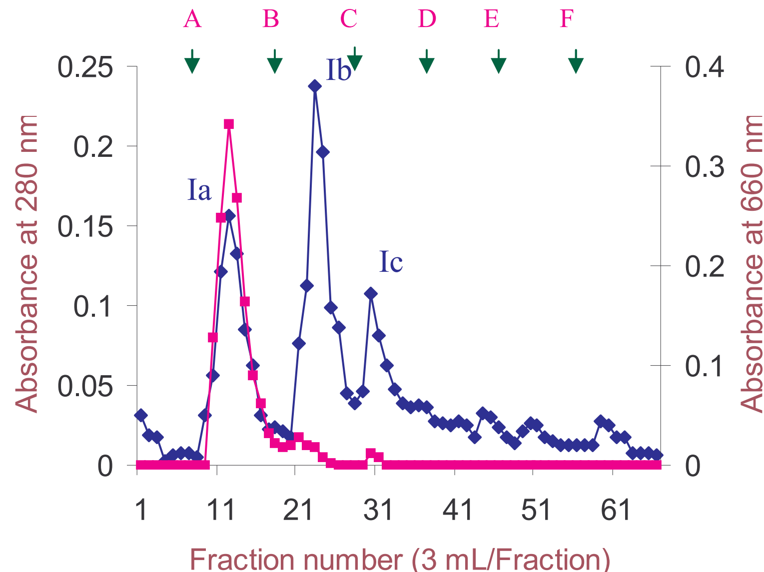



***Fig. 3.3.*** *SP-Sepharose (Fast Flow) chromatography of component I (from Sephadex G-75 purification step) by step-wise elution using different concentrations of NaCl in 50 mM sodium acetate buffer, pH 4. Arrows indicate the point of application of buffer containing the indicated NaCl concentration (M): A, 0.1; B, 0.25; C, 0.5; D, 0.75; E, 1.0; F, 1.5. Protein detection: absorbance at 280 nm (-♦-). Pentose detection (FCO assay): absorbance at 660 nm (-■-).*

### 3.4.3. SDS-PAGE analysis of chromatography pools and purified STA

Pooled component I obtained from Sephadex G-75 column chromatography, which contains active STA was subjected to SDS-PAGE (reducing), and the gel pattern shows the presence of three bands all having molecular mass >40 kD. The major

intense band at ~45 kD represents the abundant storage protein of potato, namely, patatin (Shewry 2003).

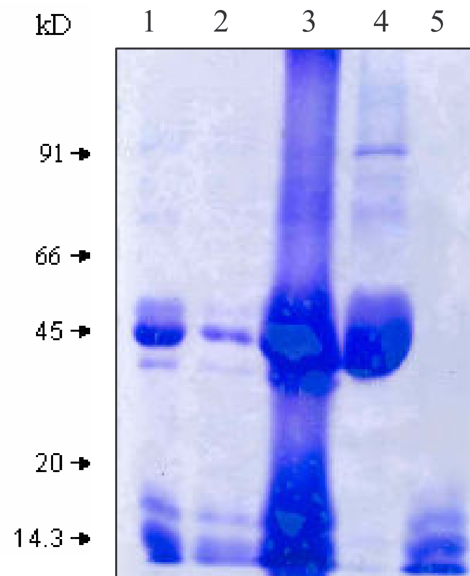



***Fig. 3.4.*** *SDS-PAGE (12%, reducing) of potato extract and certain chromatographic components from Sephadex G-75 gel filtration. The mol. wt. of marker proteins are indicated in kD on the left. Coomassie-stained gel. Lane 1, 60% ammonium sulfate precipitate of potato extract, 15 µg; lane 2, 50% (w/v) potato tuber extract, 10 µg; lane 3, ultrafiltration retentate of resolubilized ammonium sulphate precipitate, 25 µg; lane 4, component I of Sephadex G-75 (Fig. 3.2), 15 µg; lane 5, component II of Sephadex G-75 (Fig. 3.2), 10 µg. The major band near 45 kD in this gel pattern represents the major storage protein, patatin (Shewry, 2003).*

The proteins eluted from SP-Sepharose FF column (component Ia which is eluted at 100 mM NaCl concentration and component IIa which elutes at 250 mM NaCl concentration) were subjected to SDS-PAGE. The purified STA (pooled component Ia) shows a single diffuse band on 12% SDS-PAGE (reducing) in the molecular mass range of 90 to 100 kD (Fig 3.5) which is in good agreement with the anomalous behavior of this glycoprotein reported previously (Kieliszewski et al., 1994, Allen and Neuberger, 1973). The analysis of component IIa shows an intense doublet band at 45 kD which represents the major storage protein patatin (Fig 3.5).

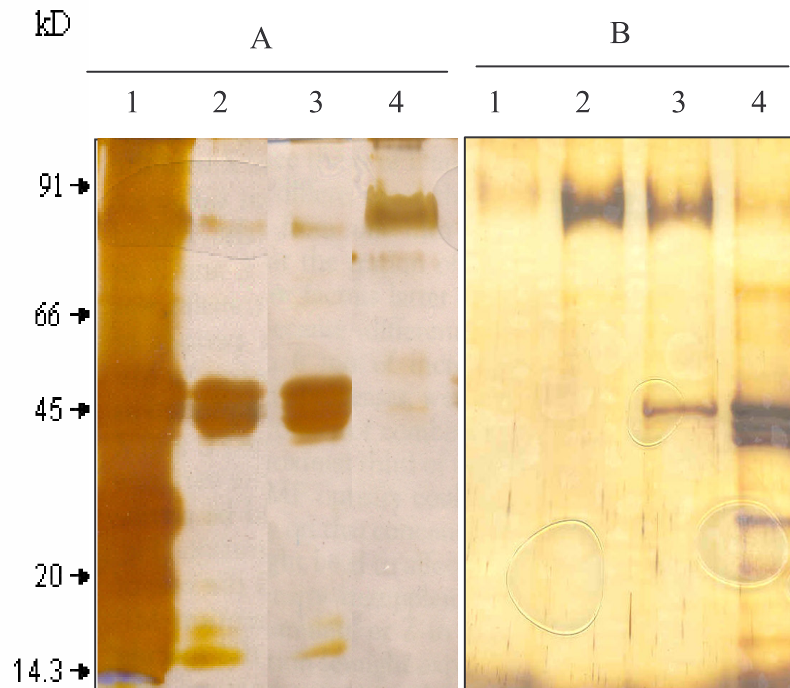



***Fig. 3.5.*** *SDS-PAGE (12%, reducing) of potato extract and certain chromatographic components from SP-Sepharose cation-exchange chromatography. The mol. wt. of marker proteins are indicated in kD on the left. Silver-stained gel.* ***A****: lane 1, concentrated potato extract, 20 µg; lanes 2 and 3, component Ib, 10 µg; lane 4, component Ia, 10 µg.* ***B****: lane 1 and 2, component Ia, 5 and 10 µg; lane 3, Fraction number 20; lane 4, 50% raw potato extract, 10 µg. The components refer to the elution pattern of SP-Sepharose cation-exchange chromatography (Fig. 3.3).*

### 3.4.4. Periodic acid-Schiff (PAS) staining and RP-HPLC for purified STA

On periodic acid-Schiff (PAS) staining for glycoproteins, purified potato lectin showed up as a single, slightly diffused pink band against a clear background (Fig 3.6), which confirms it as a glycoprotein. As a control non-glycoprotein, BSA was used which gave a negative PAS staining. Analytical reverse-phase HPLC profile, shown in Fig 3.7, revealed a major peak for the purified potato lectin with a retention time of 20.4 min and was found to be approximately 95% pure.

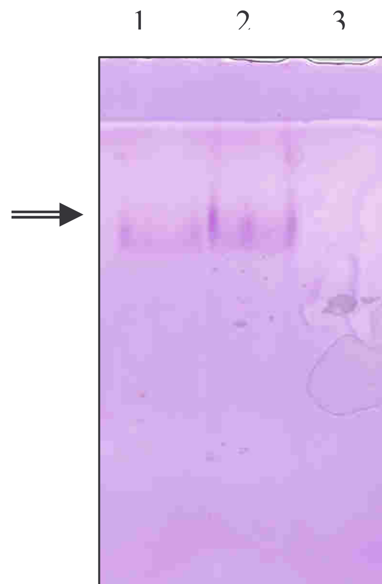



***Fig. 3.6.*** *Periodic acid-Schiff (PAS) staining of purified potato lectin. Lane 1, component Ia, 10 µg; lane 2, component Ia, 20 µg; lane 3, bovine serum albumin (BSA), 20 µg. Component Ia refers to the first component eluted in SP-Sepharose chromatography (step elution A from Fig. 3.2)*

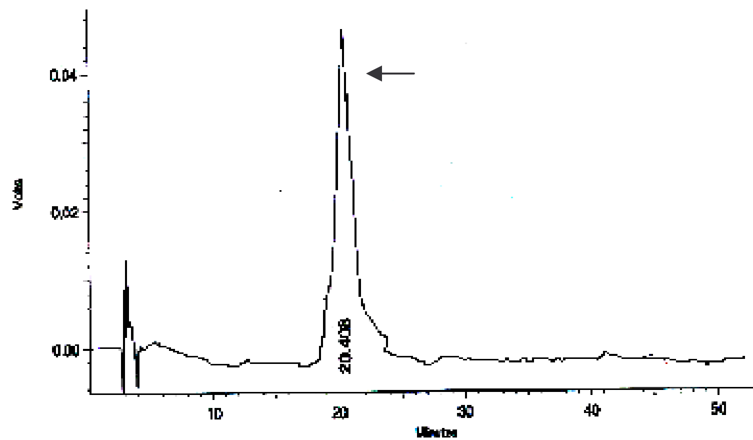


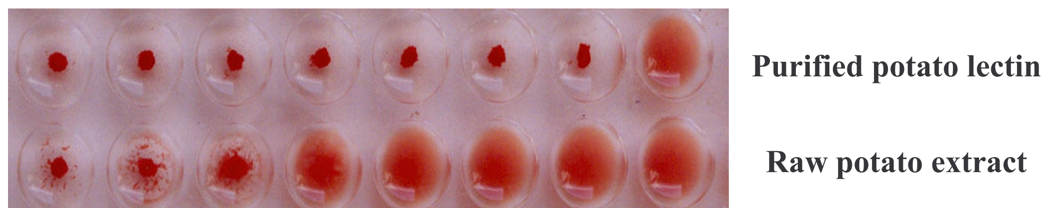

***Fig. 3.7.*** *Reverse-phase HPLC ($C_{18}$) profile of purified potato lectin (component Ia of Fig. 3.2). Protein load: 1.6 µg in a volume of 20 µL. Protein detection: 230 nm. The arrow represents the major peak with a retention time of 20.4 min.*

### 3.4.5. Hemagglutination activity

The specific activity of potato lectin as measured by hemagglutination, and the resultant fold-purification at various steps are summarized Table 3.1. The specific HA activity of potato lectin increased from 59 units/mg in crude extract to 3891 units/mg in the final step of purification (component Ia of the second chromatographic step). This translates to 66-fold purification for potato lectin after ammonium sulfate precipitation and only two chromatographic steps (Fig. 3.8). The yield of potato lectin was approximately 1.73 mg from 100 g potato tubers.

**Table 3.1. Purification of potato lectin from potato tubers (100 g)**

| Purification step | Volume (mL) | Total HA activity (Units) | Total protein (mg) | Specific HA activity (Units/mg) | Recovery (%) | Fold purification |
|---|---|---|---|---|---|---|
| Potato crude extract (50% w/v) | 200 | 8000 | 136.0 | 58.8 | 100.0 | 1.0 |
| Ammonium sulfate precipitate (60% saturation) | 25 | 7468 | 121.5 | 61.7 | 93.4 | 1.0 |
| Post-dialysis and ultrafiltration | 3 | 7202 | 43.9 | 164.2 | 90.0 | 2.8 |
| Sephadex G-75, pooled peak I | 28 | 6750 | 10.8 | 625.0 | 84.4 | 10.6 |
| SP-Sepharose FF, pooled peak Ia | 21 | 6692 | 1.7 | 3891.0 | 83.7 | 66.2 |


**Purified potato lectin**

**Raw potato extract**

***Fig. 3.8.*** *Hemagglutination of purified potato lectin and raw potato extract at 0.1 mg/mL concentration*

### 3.4.6. Ferric-orcinol assay

The detection of potato lectin in various purification steps was also followed by FCO assay in parallel, and the results were given in Table 3.2. A 16-fold increase in absorbance at 660 nm is seen in the case of component Ia (purified STA) compared to the crude extract of potato, for a constant amount of protein (50 µg).

**Table 3.2. Ferric chloride-orcinol (FCO) assay for the detection of potato lectin at various steps of its purification**

| Sample or component from a purification step (50 µg each) | $A_{660}$ | Fold-increase in absorbance* |
|---|---|---|
| Potato crude extract (50% w/v) | 0.036 | 1.00 |
| Ammonium sulfate precipitate (60% saturation) | 0.063 | 1.75 |
| Post-dialysis and ultrafiltration | 0.078 | 2.20 |
| Sephadex G-75, pooled | | |
| Peak I | **0.147** | **4.10** |
| Peak II | 0.019 | -- |
| SP-Sepharose FF, pooled | | |
| Peak Ia | **0.573** | **15.90** |
| Peak Ib | 0.012 | -- |
| Peak Ic | 0.008 | -- |

*for a constant amount of protein (50 µg)

FCO assay was carried out using free sugars (pentoses and hexoses), various non-glycoproteins and glycoproteins. The results are shown in Table 3.3. The non-glycoproteins tested (BSA, lysozyme, and concanavalin A) at 50 µg were all found to be negative in this assay. The glycoproteins containing the *N*-linked glycans (patatin, avidin, ovalbumin, HRP, soybean agglutinin, and *Dolichos biflorus* agglutinin) were also found to be negative. With the exception of soybean agglutinin, the other glycoproteins exhibited only slight positivity compared to non-glycoproteins (Table 3.3). Only potato and tomato lectins were strongly positive in this assay.

**Table 3.3. Ferric-orcinol assay for sugars, and various purified proteins**

| Sample | $A_{660}$[a] |
|---|---|
| *Saccharides (20 $\mu$g each)* | |
| L-Arabinose | **0.743** |
| D-Xylose | **0.617** |
| D-Ribose | **0.769** |
| D-Glucose | 0.021 |
| D-Mannose | 0.034 |
| D-Galactose | 0.043 |
| Gum arabic | **0.234** |
| | |
| *Protein (50 $\mu$g each)* | |
| Lysozyme | 0.007 |
| Concanavalin A | 0.002 |
| Bovine serum albumin | 0.005 |
| | |
| **Glycoprotein (50 $\mu$g each)** | |
| Patatin[b] | 0.009 |
| Soybean agglutinin | 0.007 |
| Avidin | 0.023 |
| Ovalbumin | 0.029 |
| *Dolichos biflorus* agglutinin | 0.042 |
| Horseradish peroxidase | 0.067 |
| Potato lectin[b] | **0.573** |
| Tomato lectin | **0.585** |

[a]Mean of triplicate anlaysis
[b]Proteins purified from potato in this study

### 3.4.7. Hemagglutination inhibition by sugars and glycoproteins

The inhibition in HA activity of potato lectin by GlcNAc, chitosan and its oligomers is shown in Table 4. Among these, chitosan oligomers were found to be the most potent inhibitors of agglutination. Among the proteins/glycoproteins tested at 1 mg/mL concentration, only HRP and avidin were found to be inhibitory at a concentration of 6.25 µg and 12.5 µg respectively. This may be due to the binding of potato lectin to the core $(GlcNAc)_2$ of HRP (21% glycosylation) (Takahashi et al., 1998), and avidin (13% glycosylation) which is rich in N-glycans.

**Table 3.4. Inhibition of hemagglutination activity of potato lectin by chitosan, chitosan oligomers, and selected proteins**

| Inhibitor | Inhibitory concentration (μg) |
|---|---|
| D-Glucosamine | not inhibitory |
| *N*-Acetyl-D-glucosamine | 2000 |
| Chitosan | 15.80 |
| Chitosan oligomers¶ | **4.80** |
| Horseradish peroxidase | **6.25** |
| Avidin | **12.5** |
| *Dolichos biflorus* agglutinin | not inhibitory |
| Ovalbumin | not inhibitory |
| Bovine serum albumin | not inhibitory |
| Lysozyme | not inhibitory |

¶Chitosan oligomers were obtained from digestion of chitosan (500 mg) with a non-specific enzyme pepsin (5 mg) at pH 5, 45°C for 6 h, followed by neutralization and centrifugation. Lyophilized supernatant represents chitosan oligomers (Vishu kumar and Tharanathan, 2004). The composition of chitosan oligomers as analyzed by HPLC is given under 'Results' in section 3.3.7.

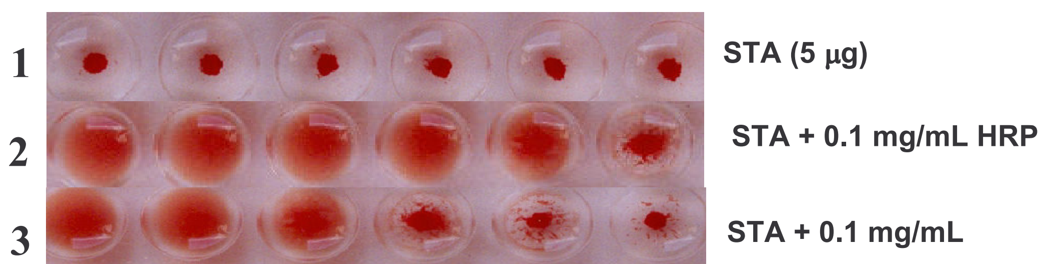



***Fig. 3.9.*** *Hemagglutination inhibition induced by HRP and avidin when used at initial concentration of 0.1 mg/mL following 2 fold serial dilutions. STA was used at 2 μg to all the wells.*

**3.4.8. Composition of chitosan oligomers**

This sample of chitosan oligomers obtained by non-specific enzymatic hydrolysis using pepsin (Vishu kumar and Tharanathan, 2004) was found to contain 78.2% of

chitotetraose, 1.6% of chitotriose, 0.9% of chitobiose, 18.2% of GlcNAc, and 1.1% of GlcN as analyzed by HPLC on an aminopropyl column (Fig. 3.10).

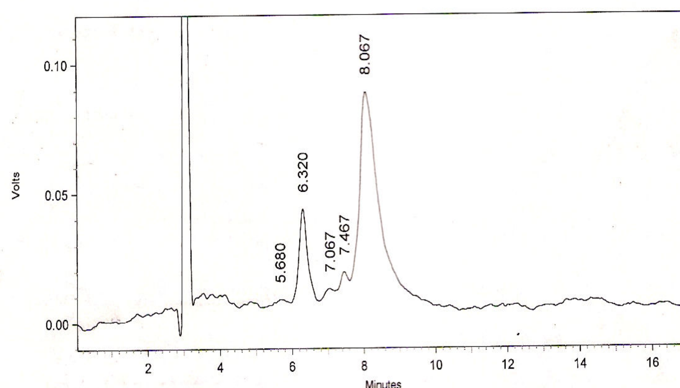



***Fig. 3.10.*** *HPLC profile of the chitosan oligomers obtained after non-specific enzyme hydrolysis of chitosan using pepsin.*

### 3.4.9. Glycoprotein binding assay for STA and LEA and its inhibition

In order to confirm the binding of potato lectin to the core $(GlcNAc)_2$ of HRP and avidin, we used glycoprotein-binding assay in a microtiter plate ELLSA format, and the results are shown in Table 3.5.

**Table 3.5. Glycoprotein-binding assay for potato lectin and its inhibition by chitosan and chitosan oligomers[a]**

| Protein coated (10 μg) | Glycoprotein binding assay $A_{492}$ or $A_{405}$[a] | |
|---|---|---|
| | HRP (0.1 mg/mL) | Avidin-AP (1:2000) |
| BSA | 0.020 | 0.013 |
| STA | 0.419 | 0.411 |
| + 50 μg chitosan[c] | 0.198 | 0.260 |
| + 50 μg chitosan oligomers[d] | 0.137 | 0.071 |
| LEA | 0.413 | 0.395 |
| Con A | 0.474 | 0.526 |

[a]chitosan oligomers preparation and composition are described in footnote to Table 3.4 and section 2.3.7.
[b]mean of triplicates

Both potato and tomato lectins were found to be positive in this assay, and the binding was substantially inhibited in the presence of chitosan oligomers. Based on its specificity for mannose, Con A was used as a positive control for glycoprotein binding (Fraguas et al., 2004).

### 3.4.10. Analyses of raw and heat-processed potato extracts

Analysis of heat-processed potato shows that only 43% protein is recovered upon extraction in comparison to raw potato. The HPPE extract was found to be very viscous and gelatinous possibly due to the high content of starch which partially degrades upon heat processing. This change may account for the lower recovery of protein. HPPE retains only 55% of biological activity as assessed by hemagglutination assay on a per mg protein basis (Table 3.6), indicating that approximately half of the biological activity of STA is not lost upon heat processing. Since an attempt to isolate STA from HPPE was not successful due to the gelation of starch during heat processing. We determined the STA content in HPPE based on glycoprotein binding assay (HRP) using a known amount of purified STA as standard. Approximately 39% of STA was present in HPPE in comparison to RPE by glycoprotein-binding assay.

**Table. 3.6. Comparison of Hemagglutination activity and HRP binding between raw potato and heat processed potato extracts**

| Samples | HA activity (Units/mg protein) | HRP binding (10 μg) ($A_{492}$) |
|---|---|---|
| RPE | 58.20 | 0.345 |
| HPPE | 29.05 | 0.214 |
| HPPE supernatant | no agglutination | 0.023 |

RPE: raw potato extract; HPPE: heat processed potato extract (Preparation described under “Methods” in § 3.2.8)
$A_{492}$ for 10 μg was 0.419.

### 3.4.10. Purification of STA from RGE and HPPE using SP-Sepharose FF

The raw and heat-processed potato extracts prepared in 50 mm sodium acetate buffer, pH 4.0 were subjected to cation-exchange chromatography on SP-Sepharose FF column. The profiles are shown in Fig 3.11.

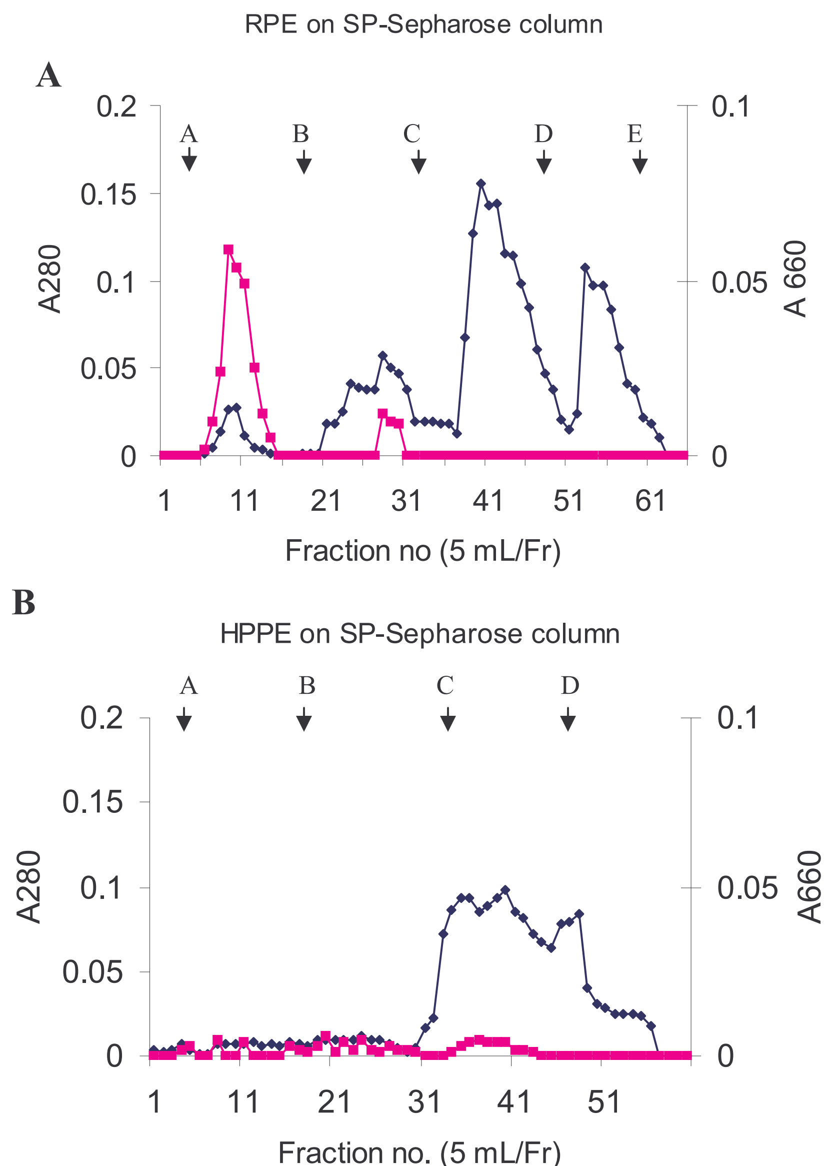



***Fig. 3.11.*** *Isolation of potato lectin from raw potato extract (RGE) (**A**) and heat-processed potato extract (HPPE) (**B**) by SP-Sepharose column chromatography. The elution was carried out step-wise using different concentrations of NaCl in 50 mM sodium acetate buffer, pH 4. Arrows indicate the point of application of buffer containing the indicated NaCl concentration (M): A, 0.1; B, 0.25; C, 0.5; D, 0.75; E, 1.0. Protein detection: absorbance at 280 nm (-♦-). Pentose detection (FCO assay): absorbance at 660 nm (-■-).*

Elution was carried out using step-wise increase of NaCl concentration from 0 to 1 M. The eluted fractions were monitored by ferric-orcinol assay to detect the presence of potato lectin. Raw potato extract showed elution of potato lectin at 100 mM NaCl concentration as observed previously during the final step of purification of STA (in the earlier purification step (section 3.3.2.2).

The isolation of potato lectin from heat processed potato extract was attempted unsuccessful following the same procedure described above for raw potato extract. In the case of HPPE, there was a significant loss in the total protein though it retained a considerable amount of lectin which was confirmed by HA and HRP binding assays. The extract prepared was very viscous which did not flow through the column easily; instead it formed a layer over the top of column bed. The eluted fractions were found to be negative by ferric-orcinol assay. Due to these technical difficulties, the isolation of potato lectin from heat processed potato extract was not successful.

### 3.5. DISCUSSION

The application of the pentose colorimetric assay for the detection of potato lectin, an arabinose-rich glycoprotein, has been demonstrated in this study. The FCO assay procedure for the determination of free pentoses (Ashwell, 1957; Dische, 1962; Chaplin, 1986) has been applied as such for the determination of protein-bound pentoses, by taking advantage of the acid-labile nature of hydroxyproline-arabinoside glycosidic linkages. Normally, a typical agglutination assay for the determination of the biological activity of a lectin or agglutinin takes 5 to 6 h. However, the detection of potato lectin by FCO assay can be completed in approximately 1.5 h. Although the FCO assay is not a substitute for the agglutination assay, there appears to be considerable saving in time for the detection of potato lectin during the various stages of the purification. The agglutination assay can then be used to confirm the biological activity of the lectin in question at the final step of the purification. It appears that core $(GlcNAc)_2$ of N-linked glycoproteins are also potent inhibitors of hemagglutination activity

of potato lectin (as exemplified by the use of horseradish peroxidase) in addition to free GlcNAc oligomers.

Additionally, a modified purification procedure involving only two chromatographic steps beyond the 60% $(NH_4)_2SO_4$ saturation step of Allen and Neuberger (1973) has been developed here. The first step is a gel filtration step wherein potato lectin (molecular mass = 100-110 kD) elutes in the void volume along with the major storage protein, patatin. Preliminary elution pattern of this gel filtration component (component Ia) on cation-exchanger SP-Sepharose by gradient elution indicated that almost all of the potato lectin elutes at a NaCl concentration of ~100 mM at pH 4 (data not shown). However, the resolution of potato lectin from other proteins was inadequate. Hence, elution from the cation-exchanger using a step elution of 100 mM NaCl at pH 4 was attempted, and found to be optimal for the purification of potato lectin with respect to its purity and recovery.

Allen and Neuberger (1973) obtained an yield of 0.84 mg potato lectin per 100 g tubers. The yield of potato lectin prepared by chromatofocusing (McCurrach and Kilpatrick, 1986) was also similar, namely, 0.92 mg per 100 g tubers. Compared to these procedures, we have obtained an yield of 1.73 mg per 100 g tubers, which translates to an increase in yield by twofold.

The FCO assay appears to be useful for the detection of glycoproteins which are rich in pentoses. Soybean agglutinin which has 6.2% carbohydrates is negative in the assay since the oligosaccharide is solely composed of $Man_9GlcNAc_2$, and behaves similar to concanavalin A, a non-glycoprotein. Ovalbumin (3.2% glycans; oligosaccharides of hybrid or high-mannose type), *Dolichos biflorus* agglutinin (4% glycans of mannose-rich type), avidin (13% glycans of complex-biantenary type), and horseradish peroxidase (21.6% glycans; oligosaccharides of complex high-mannose type with L-fucose and D-xylose characteristic of plant glycoproteins) (Spiro, 2002; Takahashi et al., 1998) give slightly higher values in the pentose assay compared to non-glycoproteins. This appears to be due to differences in glycan percentages mostly reflecting mannose content, as well as the presence or absence of a small number of

xylose residues. It may be pointed out here that phenol-sulfuric acid assay is commonly used for the detection of all neutral sugars in glycoproteins (Ashwell, 1957), whereas the FCO assay can be used to specifically detect pentose-rich glycoproteins.

Although potato lectin belongs to the family of chitin-binding lectins (Van Damme et al., 2002), it also possesses the property of binding to core $(GlcNAc)_2$ of *N*-linked glycoproteins such as ovomucoid, and fetuin (Owens and Northcote, 1980; Van Damme et al., 2002). This has also been confirmed here by inhibition of agglutination by HRP and avidin as well as glycoprotein-binding and its inhibition by chitosan oligomers. Tomato lectin behaves similarly to potato lectin in both FCO assay and glycoprotein-binding assay, based on their identical sugar specificity and glycan compositions (Van Damme et al., 2002; Peumans et al., 2003). In a recent study, LEA (and also STA) showed similar specificity for *N*-glycans based on the preference of high mannose-type glycans (Oguri, 2005). Both STA and LEA consists of two chitin binding modules that are connected by a hydroxy proline rich glycoprotein domain; this feature helps to adopt elongated structure (Peumans et al., 2003; Van Damme et al., 2004), which enables them to approach the binding sites namely, chitobiose core (Oguri, 2005).

The FCO assay for pentoses appears to be a convenient assay for the detection of other Solanaceae (tomato, datura, etc) lectins, and glycoproteins rich in pentoses (arabinose, xylose, or ribose). Glycoproteins rich in either xylose or ribose have not been reported so far (Spiro, 2002). It may be noted here that L-arabinose is one of the few L-sugars that occurs naturally; it is widely distributed in plants in complex carbohydrate gums, hemicelluloses, and pectin. The ease of application of pentose assay should prove useful for the detection and purification of these complex carbohydrate polymers as well as several hydroxyproline-rich glycoproteins (HRGPs), which are generally *O*-arabinosylated at Hyp residues. HRGPs of the plant extracellular matrix include extensins from plant cell walls (Lamport and Miller, 1971; Kieliszewski et al., 1994a; 1994b; Dey et al., 1997), gum arabic glycoprotein (Goodrum et al., 2001), repetitive proline-rich proteins, nodulins, and arabinogalactan-proteins (AGPs) (Showalter, 2001).

Recently, glycans comprising arabinose residues have been identified as carbohydrate determinants in plant pollen allergens (Leonard et al., 2005; Brecker et al., 2005). Two novel types of *O*-glycans, not found in extensins or solanaceous lectins, have been characterized in Art v 1, the major allergen of mugwort (*Artemisia vulgaris* L.) pollen – a type III arabinogalactan characterized by a large Hyp-linked arabinogalactan composed of a short β1,6-galactan core, which is substituted by a variable number (5-28) of α-arabinofuranose residues, and mono-β-arabinosylated Hyp residues (Leonard et al., 2005); the latter constitutes a new cross-reactive carbohydrate IgE epitope in plant proteins. Structural and immunological properties of arabinogalactan polysaccharides from timothy grass (*Phleum pratense* L.) pollen have revealed an IgG4 reactivity instead of IgE reactivity in humans (Brecker et al., 2005).

Analysis of heat processed potato shows only 43% protein is recovered upon extraction in comparison to raw potato. HPPE retains only 55% of biological activity as assessed by hemagglutination assay on a per mg protein basis, indicating that approximately half of the biological activity of STA is not lost upon heat-processing. STA content has been determined in HPPE using known amount of purified STA by glycoprotein binding assay (avidin-AP) and was found to retain ~39% of STA as compared to RGE. The stability of STA, which is resistant to heat appears to be due to its high content of carbohydrate residues and disulphide bonds (Matsumoto et al., 1983).

### 3.6. SUMMARY AND CONCLUSION

Potato lectin (*Solanum tuberosum* agglutinin, STA) is an unusual glycoprotein containing ~50% carbohydrates by weight. Utilizing the pentose colorimetric assay for monitoring the presence of potato lectin, a simpler and shorter procedure for the purification of this lectin from potato tubers has been developed, and STA was purified to homogeneity. The hemagglutination inhibition assay using glycoproteins revealed its binding to chitobiose core of N-linked glycans. The heat processed potato extract (HPPE) was observed to retain ~50 % of the biological activity as compared to raw potato extract indicating its heat stability.

# Chapter 4

# Modulatory effects of potato lectin (Solanum tuberosum agglutinin) on basophils and mast cells of atopic subjects

## 4.1. INTRODUCTION

Bridging of IgE molecules on the cell surface by allergen or bivalent antibody against IgE is a necessary event for IgE-mediated basophil or mast cell degranulation (Prussin and Metcalfe, 2003; van Drunen and Fokkens, 2006; Gibbs, 2005). Apart from these, lectins can either interact with specific carbohydrates on cell-bound IgE or directly with the specific carbohydrates of cell surface glycoproteins/glycolipids (Shibasaki et al., 1992; Haas et al., 1999; Moreno et al., 2003). Such interactions release histamine and other inflammatory mediators (Gibbs, 2005). Alternatively, lectins or specific antibodies can cross-link adjacent IgE Fcε receptors present on mast cells and basophils. In all these cases, the final response is similar to the allergen-specific IgE interaction seen in IgE-mediated food allergy (Kjaer and Frokiaer, 2005). Therefore, it is important to address the role of plant food lectins in mediating non-allergic food hypersensitivity (Johansson et al., 2004) (inappropriately termed as false food allergy).

Non-allergic food hypersensitivity (Johansson et al., 2004) denotes a special type of non-immunological reaction, in which a substance in the food triggers the mast cells/basophils directly or with the involvement of non-specific IgE antibodies. In this respect, lectins are probably the best-studied food components in triggering mast cells and basophils (Kjaer and Frokiaer, 2005). The activation of these cells has been studied mostly with con A (Siraganian and Siriganian, 1975; Sullivan et al., 1975; Magro and Bennich, 1977), KM+ (mannose-specific lectin from jackfruit seeds) (Moreno et al., 2003), and other mannose-specific lectins (Shibasaki et al., 1992; Haas et al., 1999); these lectins cross-link the abundant mannose residues on *N*-glycans of IgE (Arnold et al., 2004)

Mast cells and basophils are activated by lectins since lectins bind to the carbohydrate moieties present on the IgE (Fig 4.1), which induces release of histamine and other mediators by exocytosis (Shibasaki et al., 1992). Along with histamine, other chemical mediators such as interleukins, cytokines, prostaglandins and tissue necrosis factor (TNF) are also released from basophils during exocytosis (Prussin and Metcalfe, 2003)..

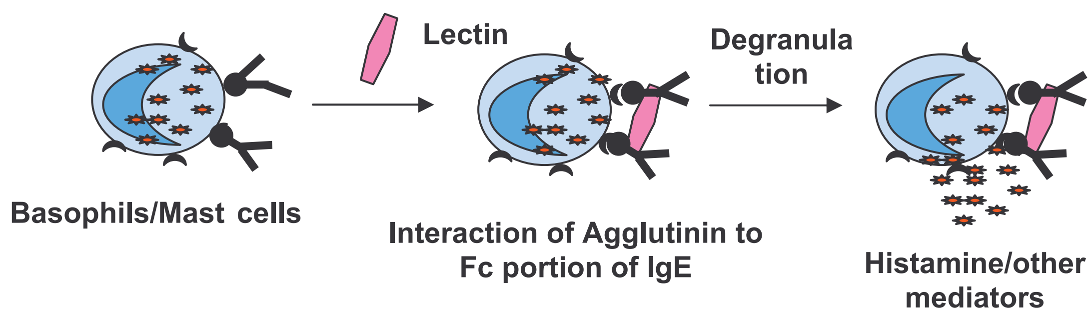



*Fig. 4.1. Lectin-mediated degranulation of mast cell/basophils, lectin interacts to the carbohydrates present on the glycans of cell-bound IgE. Bridging of two adjacent IgE by lectins causes degranulation to release inflammatory mediators*

Basophils are the least numerous of the circulating leukocytes, representing less than 1% of a typical blood differential count, they contain histamine in the preformed granules, and release after activation through their high affinity IgE receptors (Prussin and Metcalfe, 2003; van Drunen and Fokkens, 2006). One marker of systematic effects of allergic diseases is a stable basophilia in allergic subjects, and the numbers of circulating basophils increase even more (approximately 2-fold) during the allergy season (Gibbs, 2005). Thus allergic individuals have a larger pool of basophils that are capable of being recruited to sites of allergic inflammation (Bochner, 2000).

A fully differentiated mast cell, packed with 500 to 1000 granules, poised for secretion, wants only the appropriate stimulus to release the contained histamine, serotonin, heparin and hydrolytic enzymes into the connective tissue (Galli, 2000). Since the major storage site of histamine in mammalian tissue is located in the mast cell, this cell has been a major focus in the study of histamine release (Prussin and Metcalfe, 2003; Van Drunen and Fokkens, 2006).

Lectins can also bind to the lining of the gut wall and make it more leaky (Pusztai et al., 1990), so that more undigested lectins (most lectins are resistant to heat and proteases) gets into the bloodstream (Kjaer and Frokiaer, 2005). There is also some evidence that, in atopic individuals, certain lectins stimulate the body to produce IgE in preference to other antibodies (Gibbs, 2005; Haas et al., 1999). It was

demonstrated that basophils rapidly release IL-4 upon antigen-specific or non antigen-specific stimuli (certain lectins). This effect makes lectins interesting candidates for inducing a $Th_2$ response and IgE-mediated allergy in non-sensitized individuals (Haas et al., 1999; Gibbs, 2005; Kjaer and Frokiaer, 2005). All these different effects of lectins could contribute to adverse reactions to foods.

Since lectins are an important constituent of dietary proteins in many plant foods (Rudiger and Gabius, 2001), they can interact with specific sugars of *N*-glycans of cell-bound IgE. Con A was the first lectin used to study the activation of basophils and mast cells *in vitro* (Sullivan et al., 1975; Margo, 1977). In the present study, we have chosen a lectin from a widely consumed food source, namely, potato. Potato lectin (*Solanum tuberosum* agglutinin; STA) (Van Damme et al., 2004) is classified as a chitin-binding lectin (Fig 4.2); though its optimal specificity is for chitotriose and chitotetraose [$(GlcNAc)_3$ and $(GlcNAc)_4$, respectively], it binds with lower affinity to other oligomers of *N*-acetyl-D-glucosamine (GlcNAc). It also possesses the ability to bind to poly-*N*-acetyllactosamine {β-D-Gal-(1→4)-D-GlcNAc; LacNAc} moieties present in complex-type *N*-linked glycoproteins and glycosphingolipids (Kawashima et al., 1990; Ciopraga et al., 2000)

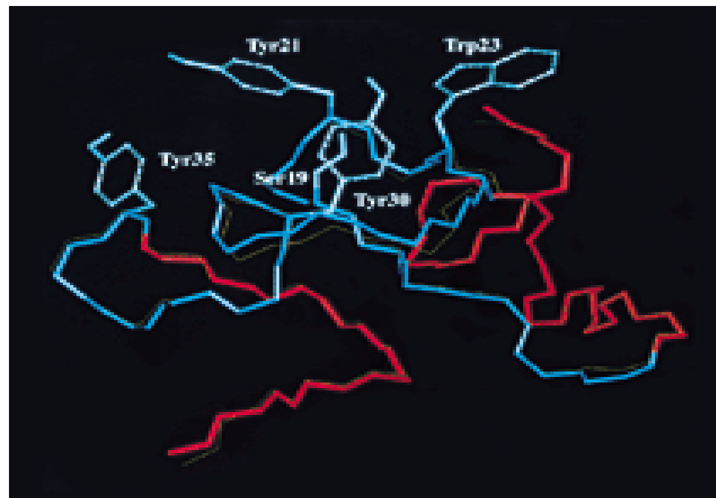



*Fig. 4.2. Model of potato lectin PL-I: Blue – portion of backbone of the known peptide sequence of PL-I. Light blue – Some amino acid side chains implicated in sugar binding (Taken from www.google/potato lectin structure, accessed on Nov 2007)*

Although the major biochemical component in potato is starch, it contains 1.6 g protein per 100 g tuber; its lectin content is ~ 6.5 mg per 100 g, which represents 0.4% of total protein (Pusztai, 2006). In view of the ubiquitous dietary importance of potato, it appeared interesting to study the effect of purified potato lectin on the degranulation of mast cells and basophils from non-atopic as well as atopic subjects.

## 4.2. MATERIALS

This study was undertaken after clearance by the Institutional Human Ethics Committee; informed consent was obtained from all atopic and non-atopic subjects in the age range of 15-60 years (for subjects below 18 years of age, consent were obtained from their parents or legal guardian).

STA was isolated from potato tubers as described in chapter 3, and found to be homogeneous by reverse-phase HPLC (95% purity; specific hemagglutination activity: 3900 units/mg). Compound 48/80, chitosan, pepsin, avidin, concanavalin (con A), *Lycopersicon esculentum* agglutinin (LEA; tomato lectin), sheep anti-mouse IgG (whole molecule)-AP conjugate, and murine anti-human IgE (monoclonal)-alkaline phosphatase (AP) conjugate were products of Sigma-Aldrich Co., St. Louis, MO, USA. Lysozyme, ovalbumin (OVA), bovine serum albumin (BSA), horseradish peroxidase (HRP) and avidin-AP were purchased from Bangalore Genei, Bangalore, India. Flat-bottom 96-well microtiter plates (MICROLON) were bought from Greiner Bio-One GmbH, Frickenhausen, Germany. All other chemicals/reagents used in this study were of analytical grade.

## 4.3. METHODS

### 4.3.1. Identification of atopic and non-atopic subjects

Atopic and non-atopic subjects were identified based on case history (atopic subjects are chosen at random who had clinical symptoms of at least one allergic condition among the following: allergic rhinitis, asthma, and food allergy) of the subjects and skin prick test (SPT) results with commercial extracts of house dust mite, and

pollens. The following allergenic extracts were used for SPT for confirming the allergic status: grass pollen mix 1 (Southern grass pollen mix #1651, Bayer Corp., Spokane, WA, USA), house dust mite (*D. farinae*, 10,000 IU/mL), weed pollen mix and grass pollen mix 2 (#P28, Greer Laboratories, Lenoir, NC, USA).

**4.3.2. Eosinophil count, serum total IgE and histamine levels**

Murine monoclonal anti-human IgE antibody (murine IgG2a, κ; hybridoma cell line ATCC HB-121, designation E5BB3IIA2) was purified by hybridoma cell culture supernatant on protein A-agarose. This cell line was obtained from National Centre for Cell Science, Ganeshkhind, Pune, India. Serum total IgE (expressed as IU/mL) was quantitated (Hamilton and Adkinson Jr, 2003) using this antibody. Following TCA precipitation of serum or plasma, histamine was extracted, determined by fluorometry (Oguri and Yoneya, 2002), and expressed as ng/mL serum. Eosinophil count was carried out on whole blood and expressed as numbers per μL of blood; the normal reference value is 40-400 (Ying et al., 2002). The eosinophil count was found to be 240-350 in non-atopics and 430-860 in atopics.

**4.3.3. Skin prick test (SPT)**

In allergological studies, purified allergens (natural or recombinant) have been used for SPT in the concentration range of 20 μg/mL to 1 mg/mL. Most whole extracts are used for SPT at a maximum concentration of 10 mg/mL. Potato lectin represents 0.4% of total proteins in the tuber (Pusztai, 2006); based on the maximum concentration of potato extract that can be used in SPT, the lectin represents 40 μg/mL. Hence, an approximate concentration of 100 μg/mL was selected for SPT so that positive results for detection of potato lectin-sensitized subjects were not missed in the present study. STA (100 μg/mL) was prepared in 50% glycerinated-phosphate buffered saline (PBS). Glycerinated-PBS was used as negative control and histamine base (1 mg/mL) was used as positive reference standard. SPT was carried out as described previously

(Sanico et al., 2002). After 20 min, the wheal/flare diameters were measured; a wheal diameter of >3 mm was considered as positive.

### 4.3.4. ELISA for detection of STA-specific IgE

STA-specific IgE was detected by indirect ELISA (Hamilton and Adkinson Jr, 2003). Briefly, microtiter wells were coated with 30 μg of STA at pH 9.6 at 4°C overnight. After the blocking step, the wells were incubated with subjects' sera at 1:3 dilution in PBS containing 1% BSA/0.05% Tween-20 at 4°C overnight. Next, it was incubated with murine monoclonal anti-human IgE-AP conjugate at 1:1500 dilution at 37°C for 2 h, followed by color development.

### 4.3.5. Isolation of leukocytes and peritoneal exudate cells

The buffy coat (leukocyte layer containing basophils) was isolated from 10 mL of venous blood as described (Kampeu et al., 1997) using 6% dextran T 700 gradient. The buffy coat was washed 4-5 times with isotonic PBS and resuspended in 10 mM Tris-HCl buffer, pH 7.4 containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.03% BSA (Tris-CAM). The isolated leukocytes were counted using crystal violet. Percentage viability of leukocytes in the buffy coat was determined by Trypan blue dye exclusion.

### 4.3.6. Isolation of rat peritoneal exudate cells (PEC)

PECs were isolated from male Wistar rats (adult; 4 weeks-old; weighing ~250-300 g) following the standard procedure (Sullivan et al., 1975) using Tyrode buffer, pH 7.4 containing 0.1% BSA. After injecting the peritoneal cavity, the fluid containing PECs was collected after 5 min, washed, and finally resuspended in Tris-CAM. PECs were stained for mast cells using toluidine blue and viability was assessed by Trypan blue dye exclusion. The PEC preparation contained 15-20% mast cells.

#### 4.3.7. Histamine release (HR) assay

Cells and reagents (STA or other proteins) in Tris-CAM were added to polystyrene tubes (final volume: 1 mL) in an ice bath. Each tube containing $2 \times 10^6$ cells/mL was incubated at 37°C for 45 min (Kampeu et al., 1997). In each experiment, addition of 3% perchloric acid or boiling at 100°C for 10 min for one set of tubes was performed to obtain the total histamine content of cells (Pc). Blank tubes containing only cells and buffer were used for non-specific release (Ps) during the reaction (generally <10%). After 45 min, the tubes were transferred to an ice bath and centrifuged at 275 x g at 4°C for 20 min. The supernatants were assayed for histamine content (Pt).

The released histamine was quantitated by a fluorometric assay (Siegel et al., 1990); the fluorescence intensity was measured using 360 nm for $\lambda_{ex}$, and 450 nm for $\lambda_{em}$. The formulae for the calculation of percent HR is $[(Pt - Ps) \div (Pc - Ps)] \times 100$, where Pt = test release, Ps = spontaneous release, and Pc = complete release.

#### 4.3.8. Histamine release assay by HPLC method

Histamine release assay was carried out as explained above for the fluorometric method, then the released histamine was condensed with *O*-pthalaldehyde to form a fluorescent condensation product and assayed by HPLC method.

Each test sample along with standard were prepared and assayed by HPLC (Tsikas et al, 1993) using $C_{18}$ column using a mobile phase containing solvents A and B at a ratio of 5:95. Solvent A is methanol and solvent B is a mixture of 0.2 M NaCl and methanol at 45:55. About 20 μL of each sample was injected and elution was carried out at a flow rate of 0.8 mL/min and monitored using a fluorescent detector at $Ex_{\lambda}$ of 355 nm and $Em_{\lambda}$ of 450 nm.

#### 4.3.9. Dot blot for glycoprotein binding assay

Dot blot (Hawkes, 1986) was carried out using STA or LEA (another lectin having similar specificity as STA), which was applied, as a spot on the nitrocellulose (NC)

membrane along with control proteins (con A and BSA). After the spots were air-dried, the membrane was blocked as described for ELISA, incubated with 0.1 mg/mL HRP at 37°C for 2 h, and color development was carried out using TMB/$H_2O_2$ substrate. The dot blot was similarly performed using avidin-AP (1:2000 dilution), and developed using BCIP/NBT.

### 4.3.10. Glycoprotein binding assay

Microtiter wells were coated with 10 μg purified STA or LEA at pH 9.6 at 4°C overnight. After the blocking step using 3% gelatin in PBS, the microtiter wells were incubated with sheep anti-mouse IgG-AP conjugate (100 μL of 1:10 dilution) in PBS containing 1% BSA/0.05% Tween-20 at 37°C for 2 h. Following the addition of the respective substrate, the absorbance was measured.

### 4.3.11. Statistical analysis

Each datum represents the arithmetic mean and standard deviation (SD) of the different experiments under identical conditions. Student's *t*-test was used to make a statistical comparison between the paired and unpaired groups. The correlation between histamine release values and serum total IgE was analyzed to find the correlation coefficient. All the data were analyzed using a computer program (Statistical Analysis System).

## 4.4. RESULTS

4.4.1. Selection of atopic and non-atopic subjects for the study

Subjects classified as non-atopic never had a history of any allergic diathesis and had negative SPTs, normal eosinophil counts, and normal IgE levels. Subjects classified as atopic had one SPT positive for any of the inhalants with a clinical history and examination suggestive of allergic rhinitis, asthma or both. Evaluation of atopic cases, including those avoiding potato ingestion, was carried out by complete clinical case history in the form of questionnaire. The atopic condition exhibiting allergic rhinitis

and asthma was confirmed by physical examination, clinical symptoms and spirometry. Based on the above criteria, the allergic status of the subjects was classified arbitrarily as non-atopic or atopic. The SPT data for allergenic extracts/HDM, total IgE, and histamine levels are summarized in Table 4.1.

**Table 4.1. Demography of the selection of non-atopic and atopic subjects for the study**

| Parameters | Subjects' status | |
|---|---|---|
| | Non-atopic | Atopic |
| Gender (M/F) | 11/9 | 53/57 |
| Wheal/flare diameter (in mm) for allergen extracts* | 0-1/0 (–) | 3-5/10-15 (1+ to 2+) |
| Wheal/flare diameter to HDM (in mm) | 0-2/0-5 (–) | 3-7/10-30 (1+ to 3+) |
| Serum total IgE (IU/mL)† | 35-44 | 59-330 |
| Plasma histamine (ng/mL)# | 1.2-2.4 | 8.6-13.4 |
| Serum histamine (ng/mL)‡ | 21-32 | 110-215 |

*Allergen extracts include various food, and commercial pollen extracts; wheal/flare diameter (range): histamine base, 5-6/20-25 mm; glycerinated PBS, 0-1/0 mm; HDM, house dust mite; SPT grading: 1+, 3-4/5-10 mm; 2+, 4-5/10-20 mm; 3+, >5/20-30 mm
†Reference normal value for serum total IgE = <120 IU/mL
#Reference normal value for plasma histamine = 0.5-2 ng/mL
‡Reported range for serum histamine (normal subjects) = 5–27 ng/mL

The total IgE was found to be significantly higher in atopic subjects, and represents a 2 to 8-fold increase over the value for non-atopic subjects. However, in non-atopic subjects, the values were in the reference range of normal. The serum and

plasma histamine levels were found to be ~5 to 8-fold higher in atopic subjects as compared to the values for non-atopic subjects.

### 4.4.2. Skin prick test of STA on atopic and non-atopic subjects

Table 4.2 shows the results of SPT with STA (100 μg/mL) tested on 110 atopic subjects who have generalized symptoms characteristic of allergic conditions, and 20 non-atopic subjects.

**Table 4.2. Results of SPT to STA in non-atopic and atopic subjects**

| Subjects' status | Number of subjects¶ | Subjects¶ + to STA | Percent positive | Avoidance to potato† (n) | Avoidance to potato (%) |
|---|---|---|---|---|---|
| Non-atopic# | 20 (M11, F9) | 0 | 0 | n.a.‡ | n.a. |
| Atopic (total)* | 110 | 39 (M18, F21) | 35.5 | 19/39 | 48.7 |
| Allergic rhinitis | 33 | 09 (M3, F6) | 27.3 | 05/09 | 55.6 |
| Asthma | 42 | 18 (M8, F10) | 42.9 | 09/18 | 50.0 |
| Allergic rhinitis with asthma | 35 | 12 (M7, F5) | 34.3 | 05/12 | 41.7 |

¶M, male; F, female
†subjects avoiding potato consumption / subjects positive to STA by SPT
#Healthy subjects (non-atopic) with no clinical symptoms of allergy
‡n.a., not applicable

SPT reactions as assessed by the mean values of wheal/flare diameter were mildly positive (3.5/5 mm) or moderately positive (4.5/10 mm). STA showed positive SPT in 39 out of 110 atopic subjects (35.5%). Nearly 49% of these subjects avoid consumption of potato in their diets. Based on the clinical symptoms, the atopic subjects

are sub-grouped as representing allergic rhinitis, asthma, or both. Again in the sub-groups, nearly 42 to 56% of subjects, who tested positive to STA by SPT, were found to avoid potato.

Among the atopic subjects who had the symptoms of allergic rhinitis or asthma (and who had reported the avoidance of potato in their diet), descriptive case history along with clinical interrogation of these subjects were carefully evaluated; these subjects report that the consumption of potato (approximately 50-100 g in a meal) in the symptomatic state increases their symptoms of asthma after 30 min to 2 h. They also experienced some kind of associated-gastrointestinal symptoms. At the time of the study, this subgroup of atopic subjects was under medication with decreased symptoms of asthma, but still avoided eating potato-containing foods.

The predictive value of diagnostic SPT to STA in the atopic population (n=110) was analyzed in reference to the clinical history with and without potato avoidance. The criteria considered were purely a subject self-reported history on potato consumption. The diagnostic sensitivity and specificity were found to be 44.1% and 72%, respectively; the positive and negative predictive values were 38.4% and 76.5% respectively, with an efficiency of 70%.

### 4.4.3. Total IgE and STA-specific IgE in atopic and non-atopic subjects

Serum samples of non-atopic (n = 10) and atopic (n = 10) subjects' were checked for the presence of STA-specific IgE. The data was summarized in Table 4.3. Con A (Man/Glc-specific lectin) and BSA (non-lectin protein) were used as negative controls. STA-specific IgE values for moderately STA-sensitive atopic subjects were very similar to those seen for mildly STA-sensitive atopic and non-atopic subjects. However, the total IgE level of moderately STA-sensitive subjects is ~2-fold higher than that of mildly STA-sensitive subjects, and ~3.5-fold higher than that of non-atopic subjects who are not sensitive to STA. The wheal / flare diameter in each subgroup was found proportional to total IgE levels, which also corresponds to percent histamine

release, indicating the non specific activation in atopic subjects by STA and its dependency on total IgE levels.

**Table 4.3. Summary of *in vivo and in vitro* diagnostic tests in the sub-group of atopic subjects positive to STA by SPT**

| Subjects' status | Subjects positive to STA | Wheal/flare diameter (mm)† | Total IgE* range ($A_{492}$) | STA-specific IgE ($A_{405}$)*¶ Mean ± SD | Histamine release (%) Mean ± SD* |
|---|---|---|---|---|---|
| Not sensitive to STA (non-atopic) | 0 | 0-1/0 | 0.248 – 0.314 | 0.045 ± 0.012 | 27.3 ± 2.2 |
| Not sensitive to STA (atopic) | 0 | 2-3/0-5 | 0.360 – 0.435 | 0.056 ± 0.009 | 31.6 ± 4.5 |
| Mildly sensitive to STA (atopic) | 24 | 3.5/5 | 0.372 – 0.647 | 0.078 ± 0.010 | 46.6 ± 6.1 |
| Moderately sensitive to STA (atopic) | 15 | 4.5/10 | 0.844 – 1.558 | 0.080 ± 0.013 | 70.5 ± 6.4 |

†mean value; positive control: 1 mg/mL histamine base (5-6/20-25 mm)
*n = 10 in each group, percent histamine release values are significant for mildly STA-sensitive atopic subjects at $p \leq 0.005$ and moderately STA-sensitive atopic subjects at $p \leq 0.001$ compared to non-atopic and atopic (not STA-sensitive) subjects.
¶Specific IgE for lectin control (Con A), n = 3: non-atopic, 0.056; atopic, 0.099
Specific IgE for non-lectin control (BSA), n = 3: non-atopic, 0.018; atopic, 0.020
STA-specific IgE values are not significant for mildly/moderately sensitive atopic subjects compared to non-atopic and atopic (not STA-sensitive) subjects at $p \leq 0.005$.

### 4.4.4. SDS-PAGE of proteins used in the study

The proteins STA (purified potato lectin), Con A (positive lectin control), OVA, BSA and lysozyme (non-lectin controls), which were used in the present study, were tested for homogeneity by 12% reducing SDS-polyacrylamide gel electrophoresis and found to be homogeneous single bands (Fig. 4.3).

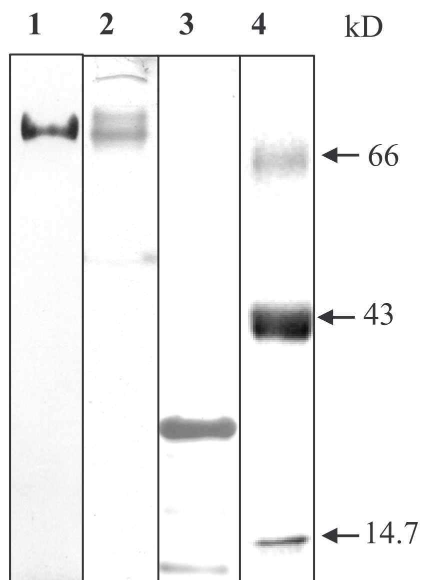



*Fig. 4.3. SDS-PAGE (12%, reducing) analyses of STA (lane 1), LEA (lane 2), and con A (lane 3). Lane 4: mol. wt. markers (BSA, OVA, lysozyme). Protein load 5 μg, the gel was stained by silver staining.*

**4.4.5. HR from non-atopic and atopic subjects using STA**

In the case of atopic subjects (n = 3), the HR assay was initially performed in the concentration range of 0.0001 to 20 μg per mL STA or the positive/negative control proteins (Fig. 4.4).

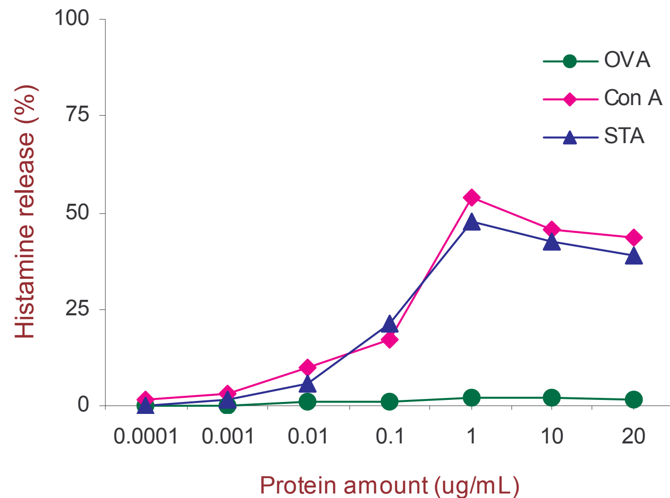



*Fig. 4.4. Histamine release from leukocytes of atopic subjects (n=3) as a function of STA concentration (0.0001 to 20 μg/mL). OVA: negative control; con A: positive control.*

The positive reference control, con A, was found to induce HR in the range of 0.01 to 20 µg/mL, and maximum release of 55% was observed at ~1 µg/mL, whereas STA showed a maximum HR of 53% at the same concentration. The non-lectin control, OVA, does not show any HR.

The HR from non-atopic and atopic subjects as a function of STA, con A, and OVA in the narrower concentration range of 0.5 to 3 µg/mL is shown in Fig. 4.5. The maximum HR was seen at 2 µg/mL in the case of both STA and con A. STA shows HR of ~28% in non-atopic (n = 5) and 67% in atopic subjects (n = 7), and the difference in HR between these two groups is significant (*P*<0.001). Con A shows a typical bell-shaped curve; HR was ~34.5% in non-atopic subjects and ~73% in atopic subjects. OVA shows HR of only 4% (non-atopic subjects) and 6% (atopic subjects); similar values were observed with BSA and lysozyme.

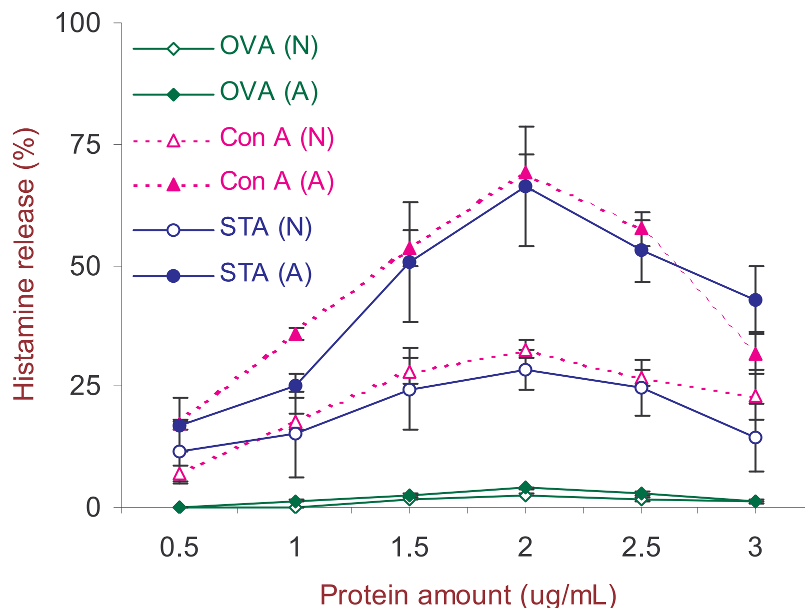



*Fig. 4.5. Histamine release from leukocytes of non-atopic (N; n = 5) and atopic (A; n = 7) subjects as a function of STA concentration (0.5 to 3 µg/mL). OVA: negative control; con A: positive control.*

**4.4.6. HR from atopic subject as determined by HPLC assay**

The pattern of histamine release measured by HPLC assay agreed well with the results from the manual extraction of histamine by fluorometric method. STA was used in the concentration range of 1-3 µg/mL. The HPLC pattern obtained at different

amounts is shown in Fig. 4.6. The maximal release was found at 2 μg of potato lectin where the peak at 7 min (representing histamine peak) shows a maximum area. The peak area of the Pc was considered as 100% release, and the Ps as standard blank. The percent histamine release was calculated and found to be in good agreement with the histamine release measured by the fluorometric method.

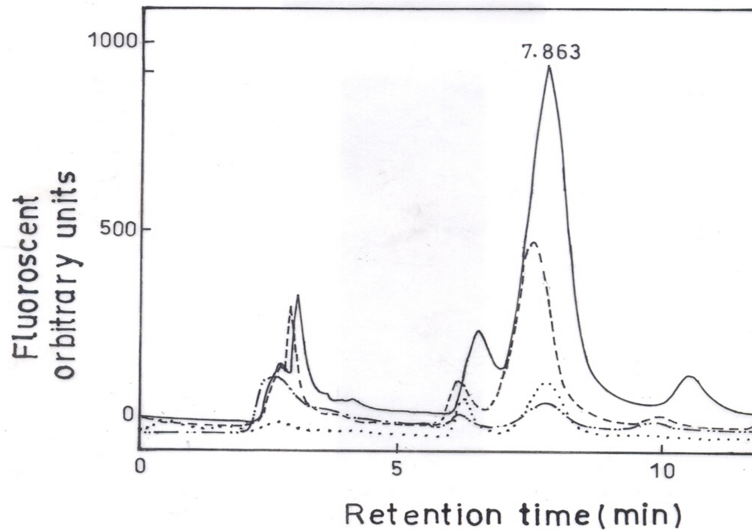



*Fig. 4.6. Histamine release from the leukocytes of atopic subject assayed by HPLC method. The histamine was detected with a retention time of 7.863 min. (_._ reagent blank, …. Ps, —— Pc, ---- STA at 2 μg)*

**4.4.6. Correlation of HR with total IgE**

Ten subjects in the non-atopic group, and 20 subjects in the atopic group were analyzed for HR and total IgE levels. The results are shown in Fig. 4.7. The percent HR was found to have a strong correlation with the serum total IgE levels ($R^2$ = 0.923, $n$ = 30). The mean value of percent HR as well as the range of serum total IgE values for both non-atopic and atopic (including its sub-groups) groups are shown in Table 4.3.

The correlation between Total IgE levels in the atopic subjects and the percent histamine release from the leukocytes isolated from the same atopic subjects, and the skin prick test data, with clinical history of subjects suggests the non-specific activation of basopils/mastcell through cell bound IgE by STA depends on total IgE levels.

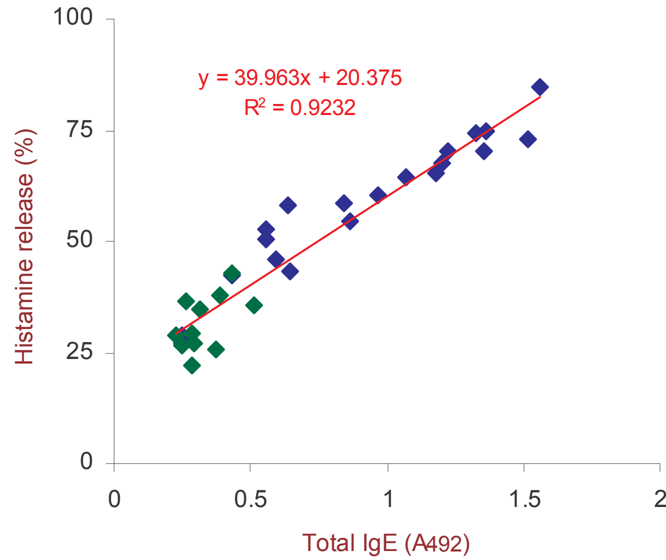



*Fig. 4.7. Correlation of leukocyte histamine release to serum total IgE (ELISA units at $A_{492}$), n = 30. (◊), non-atopic subjects (n = 10); (♦), atopic subjects (n = 20).*

**4.4.7. Inhibition of HR by chitosan and chitosan oligomers**

Since the specificity of STA is for GlcNAc oligomers, we studied the inhibition of HR from non-atopic and atopic subjects at 2 μg/mL STA in the presence of chitosan or chitosan oligomers (Fig. 4.8).

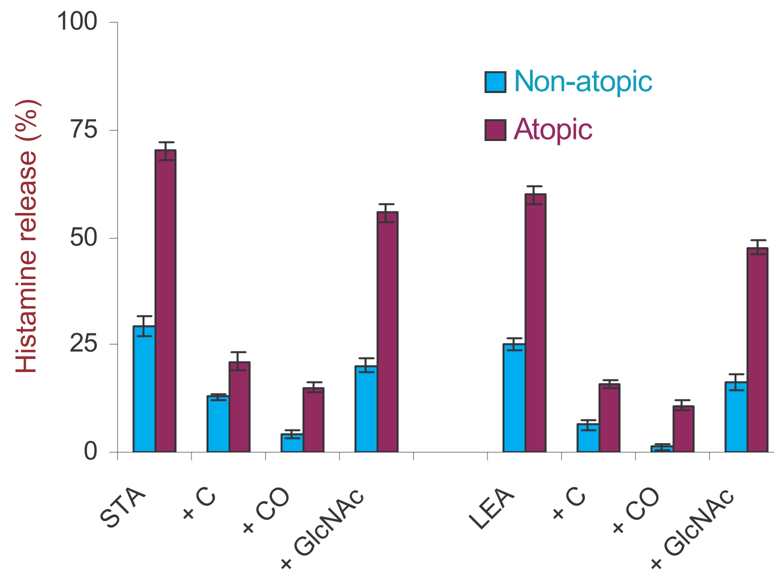



Fig. 4.8. HR from leukocytes of non-atopic and atopic subjects at 2 μg/mL STA or LEA, and its inhibition by chitosan (C), chitosan oligomers (CO), or GlcNAc at 50 μg/mL.

There is remarkable inhibition of HR in both the groups; however, among the inhibitors tested, chitosan oligomers appear to be more potent. This inhibition is significant at $P \leq 0.001$. On the other hand, GlcNAc shows only weak inhibition of HR by STA (not significant at $P \leq 0.005$). Similar results were seen in the case of LEA.

#### 4.4.8. Dot blot for glycoprotein binding

Fig. 4.9 shows the binding of HRP and avidin-AP to STA, LEA, and con A. The binding of HRP and avidin-AP to both STA and LEA was strongly inhibited by chitosan oligomers, and weakly by GlcNAc when used at 0.1 mg/mL concentration. Con A, which has specificity for mannose was used as lectin control, and was found to be positive and does not show any inhibition. Where as BSA (non-lectin control) was found negative.

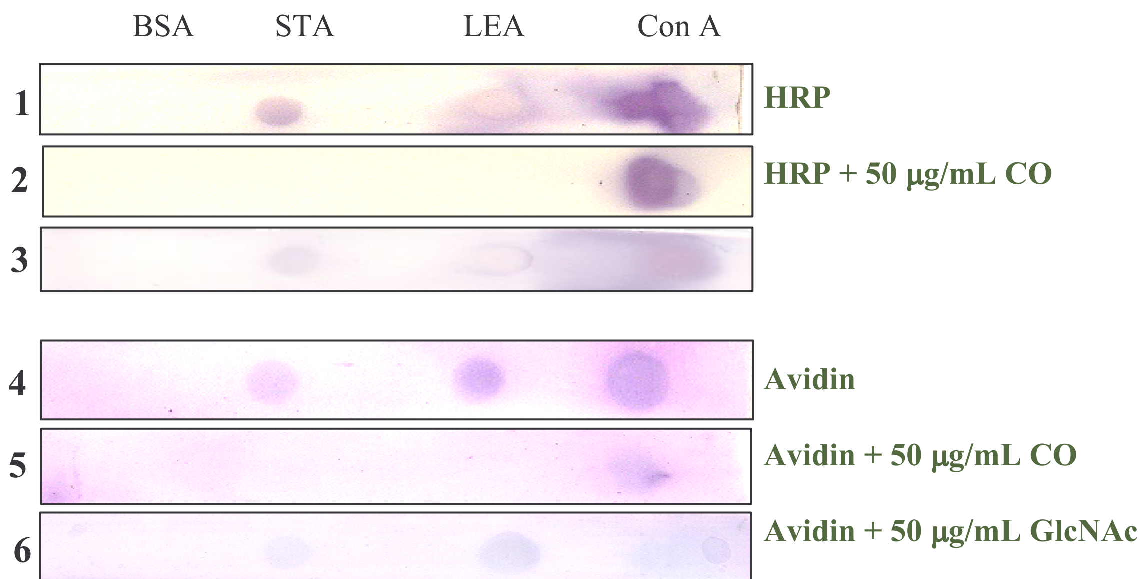



Fig. 4.9. A. Binding of STA and LEA (5 µg/spot on NC) to N-linked glycoproteins by dot-blot [rows a-c: HRP; rows d-f: avidin-AP; BSA: non-lectin control; con A: lectin control].
a: HRP alone; b: HRP + 50 µg/mL chitosan oligomers; c: HRP + 50 µg/mL GlcNAc;

d: avidin-AP alone; e: avidin-AP + 50 µg/mL chitosan oligomers; f: avidin-AP + 50 µg/mL GlcNAc.

The inhibition of hemagglutination produced by STA (at 2 μg) was studied using the glycoproteins, HRP and avidin. At 0.1 mg/mL, they were found to inhibit hemagglutination of rabbit erythrocytes at 6.25 μg and 12.5 μg, respectively (chapter 3, Fig. 3.9).

### 4.4.8. Glycoprotein binding assay for STA

The binding of STA and LEA to an IgG antibody (2-3% glycans)-enzyme conjugate was also modestly observed when sheep anti-mouse IgG-AP conjugate was used at a very low dilution of 1:10. At higher dilution the STA and LEA binding was minimum and there was no clear difference.

**Table 4.4. Glycoprotein binding assay for potato lectin and its inhibition by chitosan or chitosan oligomers**

| Protein coated (10 μg) | Glycoprotein binding assay $A_{405}$[a] IgG-AP[b] (1:10) |
|---|---|
| BSA | 0.036 |
| STA | 0.418 |
| + 50 μg chitosan[c] | n.d. |
| + 50 μg chitosan oligomers[d] | n.d. |
| LEA | 0.375 |
| Con A | 0.527 |

[a]mean of triplicates
[b]sheep anti-mouse IgG (whole molecule)-AP conjugate (0.84 mg/mL)
[c]chitosan (1 mg/mL)
[d]chitosan oligomers (prepared as given in 'Chapter 3')
n.d., not done

In order to confirm the binding of STA to core GlcNAc of *N*-linked glycoproteins, two other glycoproteins differing in their glycosylation were used. Remarkable binding of HRP (21% glycans) and avidin (13% glycans)-AP was seen in the case of both STA and

LEA compared to the non-lectin BSA (Data shown in chapter 3, Table 3.5). Both chitosan and chitosan oligomers significantly inhibited this binding. On the contrary, con A showed a similar magnitude of binding for the all the three glycoproteins indicating that mannose is abundant compared to GlcNAc in the glycan portion. It should be noted here that AP is a non-glycoprotein.

### 4.4.9. HR from rat peritoneal exudates cells

The HR from rat PECs using STA was studied in the concentration range of 1 to 5 μg/mL; the release was found to be maximum (36%) at 4 μg/mL concentration (Fig. 3, panel B). Con A shows the characteristic bell-shaped curve (data not shown) with a maximum HR of 57% at 3 μg/mL, whereas OVA, showed a maximum release of only 9% at 4 μg/mL. Compound 48/80, a known mast cell secretagogue, was used as a positive control for mast cell activation, and maximal release was observed at 10 μg/mL.

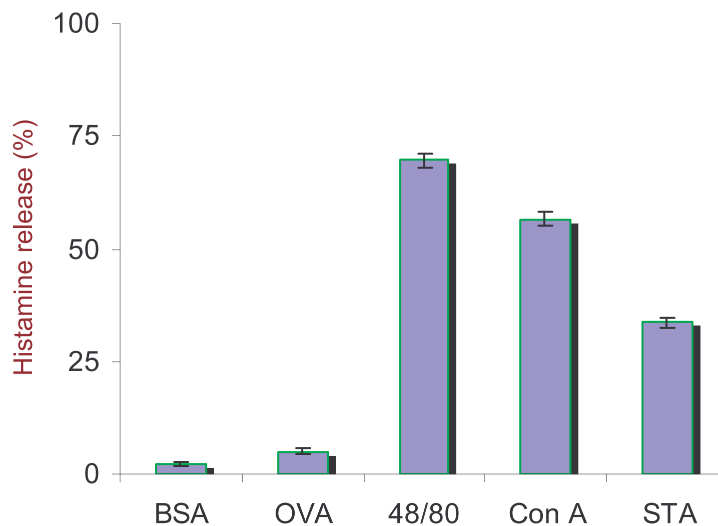



*Fig. 4.10. HR from rat peritoneal exudates cells by STA and other proteins, at 4 μg/mL. Compound 48/80 (10 μg/mL) was used as a positive control for HR from mast cells present in peritoneal exudates cells.*

## 4.5. DISCUSSION

Lectins are an important constituent of dietary proteins in many foods, especially legumes (Kjaer and Frokiaer, 2005; Rudiger and Gabius, 2001). They have been

implicated to play a role in nonallergic food hypersensitivity (Kjaer and Frokiaer, 2005). Though not a major protein, potato lectin is present in amounts of 5.2-7.8 mg per 100 g raw tubers, and ~0.5 mg per 100 g cooked material (Pusztai, 2006). The present study was undertaken to investigate the effect of a dietary lectin having specificity to GlcNAc oligomers on mast cells and basophils of non-atopic and atopic subjects; potato lectin was chosen as a prototype lectin for this specificity. This class of lectins includes the Solanaceae lectins [STA (Van Damme et al., 2004) from potato, LEA (Peumans et al., 2003) from tomato, and *Datura stromanium* agglutinin or DSA (Rudiger and Gabius, 2001) from Datura], and wheat germ agglutinin or WGA (Rudiger and Gabius, 2001) from wheat.

SPT of atopic subjects using STA revealed that ~35% of atopic subjects showed a positive reaction. However, considering that 20-25% of the general population is atopic (ISAAC, 1998), the SPT results for STA translates to 7-9% of the general population. The SPT results are unusually high for a food protein compared to the incidence of 3-4% for food allergy in adults (Prussin and Metcalfe, 2003). The sensitization rate for a major food is 0.5-1% for peanut (Mortz et al., 2005) and ~1.2% for potato in general population. This sensitization rate for potato is calculated based on the value of 5.7% for the 45 kD major allergen in potato [patatin; Sol t 1 (Seppala et al., 1999; De Swert et al., 2002)] in a study of 1886 korean patients with various allergic disorders (Lee et al., 2006). Therefore, we tried to assess the number of subjects who may have been sensitized to potato lectin by analysis of STA-specific IgE. Including this study, potato lectin has not been identified as an allergen. It should be noted that in the study by Lee et al., (2006) the potato extract concentration used for SPT is 1:20 (w/v), which is equivalent to 5%. On a protein basis, this extract contains 0.8 mg protein/mL; patatin (30-40% of total protein) and STA (0.4% of total protein) concentration in this extract amount to 0.24-0.32 mg/mL, respectively. Thus STA is present at 75-100 fold lower concentration compared to patatin in a given extract.

Interestingly it has been reported that potato lectin induces a mainly IgG (IgG1 subclass) response, and failed to induce a vigorous IgE response in BALB/c mice

(Derman et al., 2003); the authors concluded that potato lectin lacks allergenicity. Only a few lectins have been reported previously as minor allergens in four plant foods, namely, peanut (Ara h Agglutinin) (Burks et al., 1994), soybean (Gly m Lectin) (Baur et al., 1996), wheat (Tri a 18; WGA) (Wiechel et al., 2006), and horse gram (Dol b Agglutinin; DBA) (Chapter 2). The positive and negative predictive SPT values for potato lectin is in concordance with the observed positive SPT results in atopic subjects.

It has been shown that STA reacts selectively with mast cells in human connective tissue cells and epithelial cells (Roberts et al., 1990). Since cell-bound IgE is a glycoprotein rich in oligosaccharides (~12%) in its Fc portion (both oligomannose and complex bi-antennary types), the composition and structures of the *N*-linked glycans on the heavy chain of IgE (Baenziger and Kornfeld, 1974) was examined. Among the glycans of human IgE, ~86% of glycans terminate in galactose or sialic acid (Arnold et al., 2004), which represent complex bi-antennary type glycans (Baenziger and Kornfeld, 1974). Though the total IgE level was 2 to 8-fold higher in atopic subjects (who are positive to STA by SPT) as compared to non-atopics, the STA-specific IgE was found to be very similar in both atopic and non-atopic subjects confirming that none of the atopic subjects were truly allergic to potato lectin.

Although potato lectin has optimal specificity for $(GlcNAc)_3$ and $(GlcNAc)_4$ (Van Damme et al., 2004), it appears that it may also bind to the core $(GlcNAc)_2$ units found in *N*-linked glycoproteins including IgE (Taylor, 2003) (Fig. 4.11). This has been confirmed in the present study by inhibition of STA-mediated hemagglutination by *N*-linked glycoproteins such as horseradish peroxidase, avidin, and IgG, as well as inhibition of glycoprotein binding to STA by chitosan oligomers. Tomato lectin behaves similarly to potato lectin in glycoprotein binding assay, based on their structural homology and identical sugar specificity (Peumans et al., 2003).

In a recent study, LEA (and also STA) showed similar specificity for *N*-glycans based on the preference of high mannose type glycans (Oguri, 2005). Both LEA and STA consists of two chitin-binding modules that are connected by a hydroxyproline rich glycoprotein domain; this feature helps to adopt an elongated structure (Van Damme et

al., 2004; Peumans et al., 2003), which enables them to approach the binding sites, namely, chitobiose core (Oguri, 2005). The observations made in the present day on the binding of STA and LEA to *N*-linked glycoproteins strongly indicate that these Solanaceae lectins can bind to human IgE on mast cells and basophils and cross-link adjacent non-specific cell-bound IgE. Therefore, we hypothesize that STA and LEA may induce symptoms, similar to that seen in IgE-mediated food allergy in some atopic individuals.

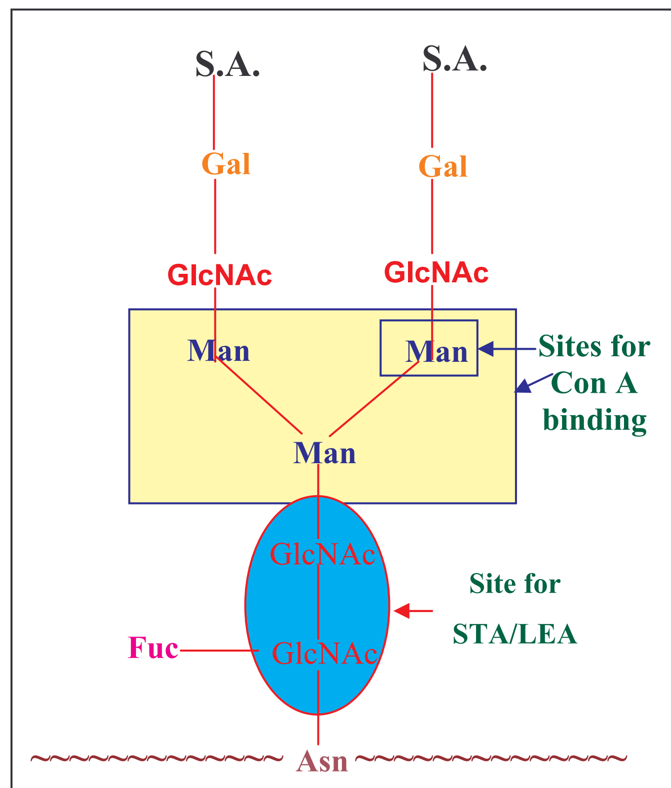



*Fig. 4.11. Structure of complex bi-antennary type N-glycan of IgE [34-35] showing the binding site for con A {specificity for mannose shown as Man (shown in a smaller box), and for trimannosidic core (shown in a larger box), and STA (specificity for GlcNAc oligomers shown as circled chitobiose). Fucose (Fuc) is absent in avidin. HRP has high-mannose type, IgE and IgG have complex bi-antennary type, and avidin has hybrid type N-glycans [12, 34-35]. One of the two branches of the IgE glycans may or may not contain sialic acid (S.A.).*

HR from the leukocytes of non-atopic and atopic subjects by STA was found to be dependent on serum total IgE levels; the release shows a strong correlation to total IgE levels ($R^2$ = 0.923). This clearly indicates that the effect of STA depends on the

basophil IgE density for its interaction in causing non-specific activation. This is strikingly similar to the effect of con A wherein the HR is higher than spontaneous release in non-atopic subjects, and comparatively more so in atopic subjects (Busse et al., 1986). It is interesting to note that a clear correlation between serum IgE and expression of Fc$\varepsilon$RI on basophils has been observed previously in allergic diseases (Saini et al., 2000). Con A-induced HR has been shown to be dependent on the density of IgE present on basophils (Margo, 1977; Busse et al., 1986). Con A binds to terminal and internal mannose on IgE glycans (Fig. 4.11), and cross-links cell-bound non-specific IgE molecules (Shibasaki et al., 1992; Haas et al., 1999) leading to degranulation.

In addition to binding to the chitobiose core of IgE, STA can also bind to the chitobiose core of the *N*-glycans of $\alpha$-chain of human Fc$\varepsilon$RI, the high-affinity IgE receptor on mast cells and basophils (Kinet, 1999). The extracellular domain of the $\alpha$-chain is heavily glycosylated (38-42% *N*-linked, and 4% *O*-linked glycans of the molecular mass of $\alpha$-chain) (Letourneur et al., 1995). The expression of Fc$\varepsilon$RI is dependent on serum IgE; since the receptor number is certainly more in the case of atopics (Busse et al., 1986; Saini et al., 2000), STA can possibly cross-link two adjacent free cell surface Fc$\varepsilon$RI through the $\alpha$-chain, and cause activation.

The stability of STA, which is resistant to heat, base, and acid, appears to be due to its high content of carbohydrate residues and disulphide bonds (Matsumoto et al., 1983). This appears to be true in the case of STA present in heat-processed potato extract, wherein ~40-50% of its biological activity (as measured by hemagglutination and glycoprotein binding assays) is retained (Chapter 3). The observation made in this study on the activation of basophils *in vitro* may also occur *in vivo*, although to a lesser extent, in view of the retention of biological activity in cooked potato. It is important to note here that tomato lectin, which is structurally and functionally similar to STA, has been shown to resist digestion in the mammalian gastrointestinal tract (Kilpatrick et al., 1985).

STA was found to release histamine from rat peritoneal mast cells similar to, but not to the same extent as, con A (Sullivan et al., 1975). This could be due to the heterogeneity of the mast cell population. It has been shown that dermal and sub-

epidermal mast cells in the rat and mouse, and mucosal and dermal human mast cells showed very similar lectin-binding properties to each other (Roberts et al., 1990). Based on the HR from rat PECs and positive SPT to STA in ~35% of atopic subjects (or 7-9% of general population), it is concluded that the binding of STA to basophils and mast cells is primarily through its interaction with the chitobiose core of *N*-glycans of cell-bound non-specific IgE. This may explain why certain atopic subjects (suffering from allergic rhinitis, asthma, or both) experience adverse reactions upon consumption of foods prepared with potato, and avoid eating potato-based foods, though they are not truly allergic to potato. A similar clinical scenario may be occurring in the case of consumption of tomato, since tomato lectin behaves in an identical fashion as potato lectin.

### 4.6. SUMMARY AND CONCLUSION

A major factor in non-allergic food hypersensitivity could be the interaction of dietary lectins with mast cells and basophils. Since IgE contains 10-12% carbohydrates, lectins can activate and degranulate these cells by cross-linking the glycans of cell-bound IgE. This study focuses on the effect of potato lectin (*Solanum tuberosum* agglutinin; STA) for its ability to release histamine from basophils *in vitro* and mast cells *in vivo* from non-atopic and atopic subjects. SPT on 110 atopic subjects using STA showed 39 subjects positive (35%); however, none showed STA-specific IgE; among 20 non-atopic subjects, none were positive by SPT. Maximal histamine release was found to be 65% in atopic subjects (n=7) as compared to 28% in non-atopic subjects (n=5); the release was specifically inhibited by oligomers of *N*-acetylglucosamine and the release correlates well with serum total IgE levels ($R^2$ = 0.923). Binding of STA to *N*-linked glycoproteins (horseradish peroxidase, avidin, and IgG) was positive by dot blot and binding assay. Since potato lectin activates and degranulates both mast cells and basophils by interacting with the chitobiose core of IgE glycans, it is speculated that higher intake of potato may increase the clinical symptoms as a result of non-allergic food hypersensitivity in atopic subjects.

# Chapter 5

# Modulatory effects of mannose-specific lectins from garlic (Allium sativum) on the immune cells involved in atopy

## 5.1. INTRODUCTION

The majority of the well-characterized plant lectins have been isolated from the seeds of dicotyledonous species. But lectins of non-seed origin from other species are also emerging as promising tools chiefly because of two reasons: (i) a good number of them might contain novel sugar-binding sites; and (ii) they can provide valuable information regarding the biological roles of plant lectins, which still remain elusive. There have been several reports of non-seed lectins from monocotyledonous families (Cammue et al., 1986; Van Damme et al., 1987a), especially Amaryllidaceae. Van Damme et al., (1991) examined a number of species including *Allium sativum* (garlic) from the family Alliaceae (taxonomically close to the family Amaryllidaceae) and found them to accumulate mannose-binding lectins. The structurally and evolutionary-related monocot mannose-binding proteins comprise a superfamily of mannose-binding lectins. Amaryllidaceae (Van Damme et al., 1987a), Alliaceae (Van Damme et al., 1991), Araceae (Sandhu et al., 1986), Orchidaceae (Van Damme et al., 1987b), Iridaceae and Liliaceae (Oda et al., 1987) families have been shown to possess these bulb lectins (Barre et al., 1996). The most remarkable property of these lectins is their high degree of stereospecificity for mannose (Shibuya et al., 1988; Van Damme et al., 1988) unlike other mannose/glucose-specific dicotyledonous legume lectins and the C-type mannose-binding animal lectins.

Garlic is the second most important bulb crop after onion. It has long been recognized all over the world as a valuable spice for food (CSIR, 1948; Gruber and Bjork, 1991). Garlic is consumed raw as well as dried in the spice form and as an ingredient to flavor the various vegetarian and non-vegetarian dishes. Good tasty pickles, chutneys, curry powders are prepared from garlic cloves. Garlic is also used to disguise the smell and flavor of salted meat and fish. Dehydrated garlic in powdered or granulated form is being used in place of fresh bulbs in many countries (Ambrose and Sreenarayana, 1998). Garlic has high nutritive value and is rich in proteins, phosphorus, potash, calcium, magnesium and carbohydrates (Gopalan et al., 2000).

Garlic (*Allium sativum L*) has long been cultivated in India as an important spice or condiment crop (Nagourney, 1998; Walker, 1996). It is a hardy perennial with narrow flat leaves, and bears small white flowers and bulbils. The bulb consists of several smaller bulbs called 'cloves', and is surrounded by a thin white, or pinkish sheath (CSIR, 1948). Garlic has a stronger flavor than onion or its other allies. No distinct varieties are cultivated. Garlic grows under much the same conditions as the onion. It requires a cool moist period during growth and relatively dry period during the maturation of the crop. It takes 4-5 months longer than onion to mature, and consequently, grown as a late season irrigated crop (CSIR, 1948; Fenwick and Hanley, 1985). The crop matures in about four months, when the leaves turn yellow and the bulbs show themselves slightly above ground. The bulbs then pulled out, cured and stored.

The bulbs of garlic are known to contain two different types of proteins with unrelated biological activities (Fenwick and Hanley, 1985), which have been intensively studied during the last two decades. One of these proteins, alliinase (Nock and Mazelis, 1986), is an enzyme that typically occurs in members of the plant genus *Allium*. The second group of well-characterized garlic proteins is the mannose-binding lectins. Detailed biochemical and molecular biological studies have demonstrated that two very homologous but not identical mannose-binding lectins (25 kD) are the most predominant proteins in garlic bulbs (Dam et al., 1998). The heterodimeric *Allium sativum* agglutinin I (ASA I) contains two slightly different subunits of 11.5 and 12.5 kD, whereas the homodimeric *A. sativum* agglutinin II (ASA II) consists of 12 kD subunits (Van Damme et al., 1992). Interestingly, both homologous subunits of ASA I are derived from a single precursor containing two tandemly arrayed lectin domains, whereas the genes encoding ASA II contain only one lectin domain (Van Damme et al., 1992; Smeets et al., 1994, 1997a). In addition, a third high molecular weight lectin *Allium sativum* agglutinin ($ASA_{110}$) was isolated from the bulb with its specificity towards mannan linkages and this belongs to those phylogenetically related group of proteins which recognize complex carbohydrate structure (Gupta and Sandhu, 1997). A lectin ASAL, has been isolated from garlic leaves (Smeets et al., 1997). Characterization of ASAL and molecular

cloning of its corresponding gene indicated that the leaf-specific lectin differs from the bulb-specific lectins ASA I and ASA II with respect to its biological activities and amino acid sequences (Smeets et al., 1997a). ASAL is a dimer of two identical subunit of 12 kD, which closely resembles the leaf lectins from onion, leak and shallot. In contrast, the root lectin ASARI, which is a dimer of subunit 15 kD, strongly differs from the leaf lectin with respect to its agglutination activity (Smeets et al., 1997b).

Both lectins and alliinase are the predominant proteins in the bulbs, but the lectins are far more abundant than alliinase. To corroborate the possible roles of the lectins and alliinase, a detailed study of the expression of their corresponding genes was made throughout the life cycle of garlic plant (Smeets et al., 1997b). It has been observed that the bulb lectins ASA I and ASA II accumulate during bulb formation and disappear when the old clove is consumed by the young plant. Bulb lectins are regulated differently during the life cycle of the garlic plant. Both bulb lectins behave as genuine storage proteins that are specifically synthesized in large quantities in developing cloves. The particular pattern of ASA I and ASA II expression in garlic indicates that these bulb lectins behave as storage proteins and is in good agreement with their possible role in defense against sucking insects (Smeets et al., 1997b).

Although a vast amount of information is available on the structure (Shankarnarayanan et al., 1996; Bachhawat et al., 2001), posttranscriptional modification and carbohydrate-specificity (Chandra et al., 1999) of garlic agglutinins (ASA I and ASA II), the relevance of these lectins in hypersensitivity has not been studied. Since a mannose/glucose specific lectin Con A has been studied well and shown to interact with mast cells or basophils to induce non-specific activation of these cells (Siranganian and siranganian, 1975; Margo, 1977), it appeared interesting to study the modulatory effects of garlic lectins in relation to atopy. In the present chapter, an attempt to isolate garlic lectins from the bulbs, by a simple method, and their biological activity in raw and heat processed garlic extracts are described. The purified lectins were studied for their possible modulatory effects on the immune cells involved in hypersensitivity, namely, mast cells and basophils.

## 5.2. MATERIALS

This study was undertaken after clearance by the Institutional Human Ethics Committee; informed consent was obtained from all atopic and non-atopic subjects aged 15-60 years (for subjects below 18 years of age, consent were obtained from their parents or legal guardian).

Garlic bulbs were procured from the local grocery. Garlic powder was a product of Nilgiris, Bangalore, India. Sephadex G-75, SP-Sepharose (fast flow) cation-exchanger (wet bead size: 45-165 µm), avidin, concanavalin (con A), sheep anti-mouse IgG (whole molecule)-AP conjugate, and murine anti-human IgE (monoclonal)-alkaline phosphatase (AP) conjugate were products of Sigma-Aldrich Co., St. Louis, MO, USA. Lysozyme, ovalbumin (OVA), bovine serum albumin (BSA), horseradish peroxidase (HRP) and avidin-AP were purchased from Bangalore Genei, Bangalore, India. Flat-bottomed 96-well microtiter plates (MICROLON) were bought from Greiner Bio-One GmbH, Frickenhausen, Germany. All other chemicals/reagents used in this study were of analytical grade.

## 5.3. METHODS

### 5.3.1. Preparation of garlic extracts

***Raw garlic extract (RGE):*** Garlic bulbs (25 g) were peeled to remove the external skin (bulb coat), washed, cut into pieces, and homogenized by blending in 50 mL of (a) phosphate-buffered saline (PBS), pH 7.4 (for neutral pH buffer extract) or (b) distilled water (for aqueous extract) or (c) 50 mM sodium acetate buffer, pH 4.0 (for acidic pH buffer extract) to obtain a 50% w/v RGE. After keeping at 4°C for 2 h, the extract was initially passed through a porous gauge, and then filtered through a muslin cloth, and the filtrate was centrifuged at 5000 x *g* at 4°C for 15 min. The clear pale yellowish supernatant obtained was stored at 4ºC; the precipitate was resuspended again in the same buffer and the procedure repeated twice for complete extraction.

***Garlic powder extract (GPE)*****:** Commercial garlic powder (25 g) was suspended in 50 mL of PBS to obtain 50% w/v extract. The extract was kept at 4°C for 2 h with

stirring. The extract was then filtered through a muslin cloth, and the filtrate was centrifuged at 5000 x *g* at 4°C for 15 min. The clear light reddish supernatant obtained was stored at 4ºC.

***Heat processed garlic extract (HPGE)*****:** Twenty-five grams of garlic bulb (after removing the bulb coat) were suspended in 50 mL of PBS, pH 7.4. The contents were boiled for 20 min; later, the boiled contents were allowed to cool at room temperature (5 mL of the clear boiled solution was stored as HPGE supernatant). Then the mixture was ground using mortar and pestle to obtain HPGE. The extract was initially filtered through a porous gauge and later filtered using muslin cloth followed by Whatman No. 1 filter paper. The filtrate was then subjected to centrifugation at 5000 x g at 4° C. The clear supernatant was collected and stored at 4°C. The HPGE supernatant obtained was concentrated by refrigerated Speed VAC (concentration system, RCT 60, Jouan) concentrator and stored at 4ºC. All the above extracts were used for determining the lectin content and biological activity, as assessed by glycoprotein binding assay and hemagglutination assay, respectively.

### 5.3.2. Purification of garlic lectins from raw garlic bulbs

#### 5.3.2.1. Preparation and concentration of raw garlic extract

Raw garlic extract (aqueous extract) was prepared as explained earlier in section 5.3.1. Garlic extract (obtained from 100 g of garlic bulbs) was initially subjected to ammonium sulfate precipitation to concentrate the proteins. For precipitation, clear extract was subjected to sequential 20% and 80% ammonium sulfate saturation; $(NH_4)_2SO_4$ (10.6 g/L) was added to aqueous extract of raw garlic to produce 20% saturation at 4ºC. The precipitate was collected by centrifugation at 9000 x g for 20 min and discarded. $(NH_4)_2SO_4$ (17.5 g/L) was then added to the supernatant to produce 80% saturation at 4ºC; the precipitate was allowed to settle overnight and then collected by centrifugation at 9000 x g for 20 min. The precipitate was resuspended in distilled water, dialyzed extensively against water, and was further concentrated by lyophilization.

#### 5.3.2.2. Gel permeation chromatography on Sephadex G-50

Concentrated raw garlic extract (4 mL containing 30 mg of total protein) was loaded on to Sephadex G-50 column (1.85 x 157 cm) pre-equilibrated with distilled water. Elution was carried out with distilled water at a flow rate of 12.5 mL/h. The eluted fractions (2.5 mL/fraction) were monitored by absorbance at 280 nm, and the presence of lectin in the eluted fractions was followed by glycoprotein-binding assay.

#### 5.3.2.3. Cation-exchange chromatography on SP-Sepharose FF

The lectin containing pool (pool P3) obtained from Sephadex G-50 column chromatography was adjusted to pH 4.0 using 50 mM sodium acetate buffer, pH 4.0, and subjected to cation-exchange chromatography on SP-Sepharose FF (1 x 13 cm; column volume 10 mL) which was pre-equilibrated with 50 mM sodium acetate buffer, pH 4.0. Sample (pool P3) was loaded and the flow through was recycled twice for facilitating maximum binding of proteins. After collecting the flow-through, the column was washed with 5 column volumes of the same buffer to remove non-specifically bound proteins. Bound proteins were eluted by step-wise elution with different NaCl concentrations (steps of 0.1, 0.25, 0.5, 0.75, 1, and 1.5 M) in the starting buffer. Volume of buffer used for each step was 30 mL; the flow rate was maintained at 25 mL/h and the fraction volume was 5 mL/fraction.

### 5.3.3. Preparation of 2% rabbit erythrocytes and hemagglutination (HA) assay

Five milliliters of fresh rabbit blood was collected and put into 5 mL of Alsever's solution. The contents were mixed by swirling in order to prepare 2% RBC suspension for use in HA assay (see Chapter 3, § 3.3.3.1. for details).

HA activity of the garlic extracts and purified lectins (ASA I and ASA II) were carried out using trypsinized rabbit erythrocyte suspension as described by Burger (1974). Briefly, 2% suspension of rabbit erythrocytes (0.2 mL) was added to serially diluted garlic protein solutions. After incubation at 37°C for 1h, agglutination was visualized. The HA specific activity is given as the number of HA units per mg of protein.

#### 5.3.4. Glycoprotein binding assay

Microtiter wells were coated with 10-20 μg protein of various garlic extracts or purified garlic lectins (ASA I and ASA II) at pH 9.6 at 4°C overnight. After the blocking step using 3% gelatin in PBS, the microtiter wells were incubated with HRP (100 μL of 0.1 mg/mL), or avidin-AP conjugate (100 μL of 1:2000 dilution) in PBS containing 1% BSA/0.05% Tween-20 at 37°C for 2 h. Following the addition of the respective substrate (*o*-phenylenediamine/$H_2O_2$ for HRP or *p*-nitro phenyl phosphate for alkaline-phosphatase), the absorbance was measured.

#### 5.3.5. SDS-PAGE and protein assay

The protein pattern of the garlic extracts and selected pools from the above two chromatographic steps were analyzed by 12% or 15% SDS-PAGE (reducing), as per the procedure of Laemmli (1970). The protein bands were visualized either by coomassie blue or silver staining. Protein assay was carried out as per the procedure of Bradford (1976), using BSA as the standard.

#### 5.3.6. Selection of non-atopic and atopic subjects

Atopic and non-atopic subjects were identified based on case history (atopic subjects were chosen at random who had clinical symptoms of at least one allergic condition among the following: allergic rhinitis, asthma, and food allergy) of the subjects and skin prick test (SPT) results with commercial extracts of house dust mite, and pollens. The following allergenic extracts were used for SPT for confirming the allergic status: grass pollen mix 1 (Southern grass pollen mix #1651, Bayer Corp., Spokane, WA, USA), house dust mite (*D. farinae*, 10,000 AU/mL), weed pollen mix and grass pollen mix 2 (#P28, Greer Laboratories, Lenoir, NC, USA).

#### 5.3.7. SPT of purified garlic lectins

Purified garlic agglutinin ASA I or ASA II (100 μg/mL) was prepared in 50% glycerinated-phosphate buffered saline (PBS). Glycerinated-PBS was used as negative

control and histamine base (1 mg/mL) was used as positive reference standard. SPT was carried out as described earlier in chapter 4, § 4.3.3. After 20 min, the wheal/flare diameters were measured; a wheal diameter of >3 mm was considered as positive.

### 5.3.8. Histamine release (HR) from leukocytes using ASA I and ASA II

The buffy coat (leukocyte layer containing basophils) from atopic and non-atopic subjects was isolated from 10 mL of venous heparinized blood as described in Chapter 3, § 3.2.5 using 6% dextran T 700 gradient. Peritoneal exudate cells (PECs) were isolated from male Wistar rats (adult; 4 weeks-old; weighing ~250-300 g) following the standard procedure explained in Chapter 4, § 4.3.5.6 using Tyrode buffer, pH 7.4 containing 0.1% BSA.

Cells and reagents (ASA I and ASA II or other proteins) were incubated and the released histamine from cells was extracted following the procedure explained in chapter 2, § 2.3.9. The released histamine was quantitated by a fluorometric assay; the fluorescence intensity was measured using 360 nm for $\lambda_{ex}$, and 450 nm for $\lambda_{em}$. The formulae for the calculation of percent HR is $[(Pt - Ps) \div (Pc - Ps)] \times 100$, where Pt = test release, Ps = spontaneous release, and Pc = complete release.

### 5.3.9. Preparation of RGE and HPGE in acidic pH buffer

For raw garlic extract (RGE), 50 g of peeled garlic bulb was taken and blended in 50 mL of 50 mM sodium acetate buffer, pH 4.0. The extract was passed through a muslin cloth and then filtered using Whatman no. 1 filter. The extract was then centrifuged in the cold at 5200 x g for 15 min. For HPGE, 50 g of peeled garlic bulb was taken and suspended in 50 mL of 50 mM sodium acetate buffer, pH 4.0. The contents were boiled for 20 min. After cooling, the contents were ground using mortar and pestle at 25°C. The extract was passed through a muslin cloth and then filtered using Whatman no. 1 filter. The extract was then centrifuged in the cold at 5200 x g for 15 min.

### 5.3.10. SP-Sepharose FF cation exchange chromatography

The raw or heat-processed garlic extract prepared in 50 mM sodium acetate buffer, pH 4.0 was subjected to cation-exchange chromatography on SP-Sepharose FF column pre-equilibrated with the same buffer. The elution was carried out using step-wise increase of NaCl concentration from 0 to 1 M, as described earlier in section 5.3.2.3. The eluted fractions were monitored for biologically active lectin by HRP-binding assay.

## 5.4. RESULTS

### 5.4.1. Preparation of garlic extracts

In order to standardize the extraction method for obtaining 50% w/v extract, 25 g peeled garlic bulbs were extracted with (a) distilled water, or (b) 50 mM sodium acetate buffer, pH 4.0 or (c) phosphate-buffered saline, pH 7.2. The results are shown in Table 5.1. The amount of protein extracted was found to be: (a) 10.2 mg/g, (b) 9.6 mg/g, and (c) 11.2 mg/g. It is clear that extraction with PBS results in a slightly higher protein yield as compared to aqueous or acidic pH extracts. Garlic powder extract has a protein yield of 7.74 mg/g. Heat-processed garlic extract has comparatively very low yield of protein (0.74 mg/g), probably due to difficulty in the extraction from boiled garlic bulbs (Table 5.1). The HPGE supernatant shows almost no protein compared to other garlic extracts.

### 5.4.2. Hemagglutination activity and lectin quantitation in garlic extracts

In the glycoprotein binding assay as assessed by HRP and avidin-AP binding, the neutral pH extract shows considerable binding, and is higher compared to other extracts. GPE shows almost similar binding as that of RGE (neutral pH). The trend in hemagglutination assay is also found to be similar to that seen in glycoprotein binding assay. Magnitudes of glycoprotein binding and hemagglutination activity of the different extracts are shown in Table 5.1. HPGE shows approximately 30% reduction in the

specific hemagglutination activity compared to other extraction procedures, whereas HPGE supernatant shows only traces of lectin by glycoprotein binding assay.

**Table 5.1. Extraction of garlic proteins, hemagglutination activity and glycoprotein binding assay**

| Extraction method | Protein (mg/g)[#] | Glycoprotein binding assay* | | HA activity (Units/mg of protein) |
|---|---|---|---|---|
| | | HRP-BA $A_{492}$ | Avidin-AP $A_{405}$ | |
| RGE (aqueous) | 10.20 | 0.057 | 0.146 | 21.5 |
| RGE (acidic pH) | 9.60 | 0.050 | 0.125 | 19.4 |
| RGE (neutral pH) | 11.2 | 0.065 | 0.178 | 23.5 |
| GPE | 7.74 | 0.062 | 0.163 | 20.6 |
| HPGE | 0.74 | 0.036 | 0.072 | 14.3 |
| HPGE (supernatant) | 0.56 | 0.014 | 0.018 | no HA |

[#]Amount of garlic cloves taken for extraction 25 g
*20 μg protein coated on microtiter wells; HRP concentration: 0.1 mg/mL; avidin-AP dilution-1:1500
Values for control protein (BSA): 0.009 (HRP-BA); 0.015 (Avidin-AP)

### 5.4.3. SDS-PAGE pattern of garlic extracts

SDS-PAGE (12% reducing gel) gel pattern is shown in Fig 5.1, and the patterns are identical in all the 3 extracts. From these results, it appears that extraction with neutral pH appears to be the ideal extraction method for purification of agglutinins from garlic, since the intensity of the protein bands is somewhat higher compared to that observed for other extracts, for a constant amount of protein load.

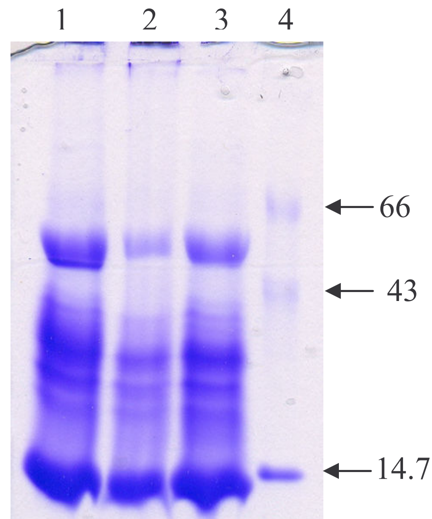



*Fig 5.1. SDS-PAGE (12% reducing)- of garlic extracts. Lane 1 – neutral pH, Lane 2 – acidic pH, Lane 3 - aqueous, Lane 4 - MW markers (top to bottom: BSA, ovalbumin, lysozyme). Protein load: 15 $\mu$g in each case.*

In the case of garlic powder extract some protein bands between 40-70 kD are not at all seen or of low intensity as compared to the pattern in raw garlic extract (Fig. 5.2). This indicates that some proteins in the mol. wt. range of 40-70 kD in raw garlic powder have been lost during the preparation of commercial garlic powder.

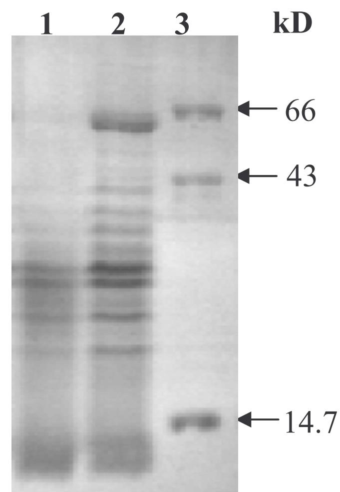



Fig. 5.2. SDS-PAGE (15%, reducing). Lane 1 – Extract from garlic powder (CGPE), Lane 2 - Extract from raw garlic (RGE-PBS), Lane 3 - MW markers (top to bottom: BSA, ovalbumin, lysozyme).

### 5.4.4. Purification of garlic lectins

The lectins were purified from garlic bulbs by conventional chromatography in two steps, namely, gel permeation (Sephadex G-50) and cation exchange (SP-Sepharose FF).

#### 5.4.4.1. Sephadex G-50 gel permeation chromatography

Sephadex G-50 chromatography pattern of garlic extract (Fig 5.3) shows that the garlic proteins are fractionated into several components, and designated as P1, P2, P3, P4 and P5 pools. The pooled component P3 was positive by glycoprotein-binding assay and hemagglutination activity, which indicates the elution of garlic lectins (ASA I and ASA II) in pool P3. Pools P1 and P2 represent complexes of alliinase with ASA I/ASA II, free alliinase and high molecular weight agglutinin ($ASA_{110}$). Pool P4 consists of low molecular weight proteins in the range 6-14 kD devoid of agglutination activity, and P5 represents very low molecular weight polypeptides along with sulfur-containing phytochemicals.

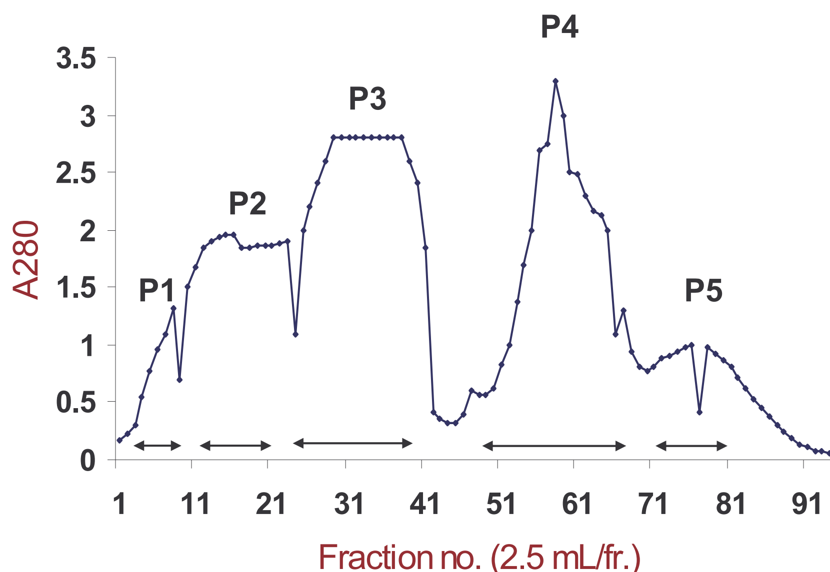



*Fig. 5.3. Gel filtration of concentrated raw garlic extract (RGE) on Sephadex G-50 (1.85 x 157 cm) using distilled water as the eluant. Flow rate: 15 mL/h. Protein detection: absorbance at 280 nm. Fractions were not collected for the initial 120 mL.*

Fig 5.4 shows the SDS-PAGE (15% reducing) pattern of Sephadex G-50 pools P1 to P5; component P3 comprising garlic lectins shows a diffused protein band at < 14 kD. Protein bands were not observed in Pool P5 (data not shown).

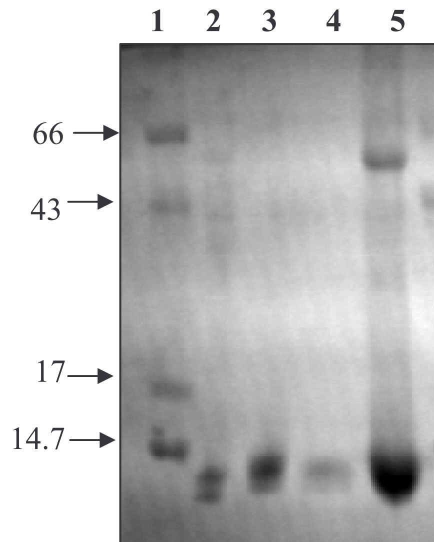



*Fig. 5.4. SDS-PAGE (15%) of Sephadex G-50 pools. Lane 1: Molecular wt. markers (top to bottom: BSA, ovalbumin, avidin, lysozyme), Lane 2: Pool 2 (P2), Lane 3: Pool 3 (P3), Lane 4: Pool 4 (P4), Lane 5: raw garlic extract (RGE).*

**5.4.4.2. SP-Sepharose FF cation exchange chromatography**

Pool P3 obtained from Sephadex G-50 gel permeation chromatography was further fractionated on SP-Sepharose FF cation exchanger by step-wise elution with NaCl at pH 4. Four components are seen, and labeled as P3a, P3b, P3c and P3d (Fig 5.5). The peak fractions of all the four components were analyzed by HRP-binding assay, and only components P3b and P3c eluting at 250 mM and 500 mM NaCl concentrations, respectively, were found to be positive. The components were pooled, dialyzed extensively against water, and analyzed by SDS-PAGE and dot blotting. Components P3b and P3c were found to possess hemagglutination activity for rabbit erythrocytes.

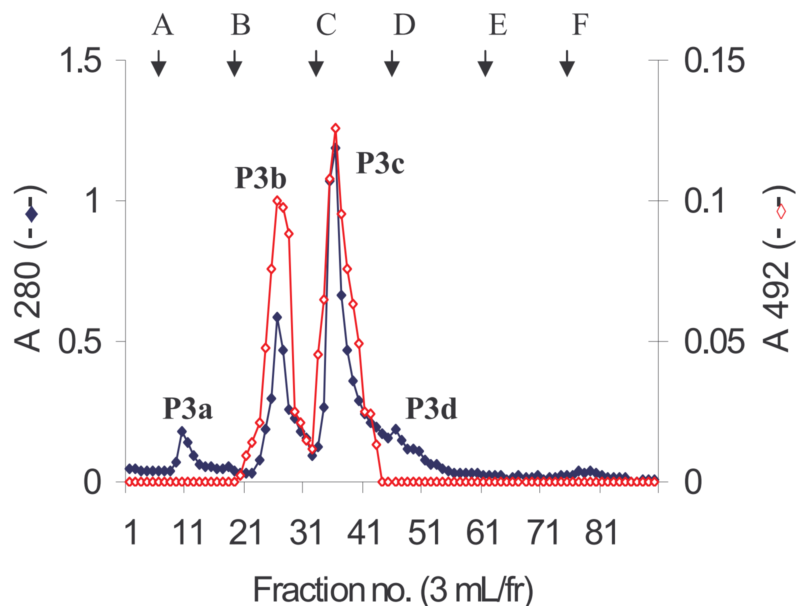



*Fig. 5.5. SP-Sepharose (fast flow) chromatography of component P3 (from Sephadex G-50 purification step) by step-wise elution using different concentrations of NaCl in 50 mM sodium acetate buffer, pH 4. Arrows indicate the point of application of buffer containing the indicated NaCl concentration (M): A, 0.1; B, 0.25; C, 0.5; D, 0.75; E, 1.0; F, 1.5. Protein detection: A280 nm (-♦-), HRP-binding: A 492 nm (-◊-).*

### 5.4.4.3. SDS PAGE and dot blot of purified garlic lectins

Components P3b and P3c were analyzed by 15% SDS-PAGE (reducing), and subjected to both coomassie blue and sliver staining for visualization. Both components appeared as a single band of slightly <14 kD in coomassie staining (Fig. 5.6) and P3c component appeared slightly diffused. Upon silver staining, component P3c resolved into a slightly doublet of < 14 kD, whereas component P3b appeared as a homogenous single band. The protein pattern of purified P3c and P3b on both coomassie and silver staining gels are shown in Fig 5.6.

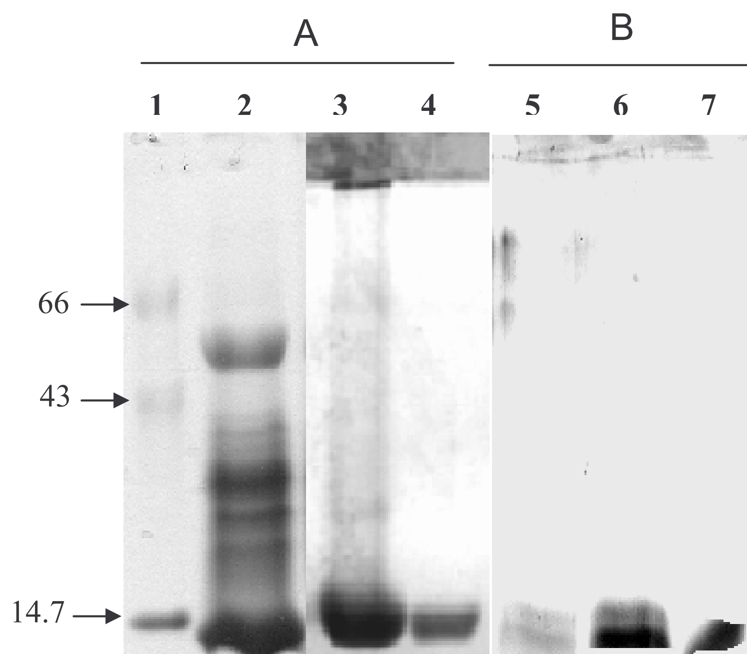



*Fig. 5.6. SDS-PAGE (15% reducing) pattern of purified garlic lectins (P3b and P3c). A. Coomassie staining; Lane 1: marker proteins (BSA, ovalbumin and lysozyme), lane 2: concentrated raw garlic extract (RGE), lane 3: component P3c, lane 4: component P3b. B. Silver staining; lane 5: component P3c (2.5 $\mu$g); lane 6: component P3c (6 $\mu$g); lane 7: component P3b (6 $\mu$g).*

### 5.4.4. Dot-blot for purified garlic lectins

Components P3b and P3c were spotted onto nitrocellulose membrane, and developed using horseradish peroxidase and avidin-AP. The results are shown in Fig. 5.7. Both components P3b and P3c were found to bind peroxidase and avidin and hence, are identified as mannose-binding lectins. The reference control for mannose/glucose binding lectin Con A was also positive, whereas the non-lectin control protein BSA was negative.

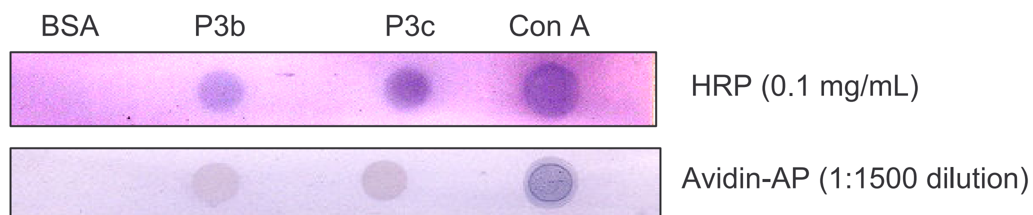



*Fig. 5.7. Dot blot to demonstrate the glycoprotein-binding nature of garlic lectins. Membrane: nitrocellulose. BSA: non-lectin control, Con A: lectin control. Amount of protein spotted: at 10 $\mu$g.*

Based on the SDS-PAGE pattern, hemagglutination and glycoprotein-binding assays, components P3c and P3b represent most likely, ASA I and ASA II respectively. The specific activity of garlic lectins as measured by hemagglutination, and the resultant fold-purification at various steps are summarized in Table 5.2.

**Table 5.2. Purification of garlic lectins (ASA I and ASA II) from garlic bulbs (100** g)

| Purification step | Total HA activity | Total protein (mg) | Specific HA activity (Units/mg) | Recovery (%) | Fold purification |
|---|---|---|---|---|---|
| Raw garlic extract (RGE) | 16817 | 784.0 | 21.5 | 100.0 | 1.0 |
| Post concentration and dialysis | 16511 | 702.0 | 23.5 | 98.2 | 1.1 |
| Sephadex G-50 (Pool P3) | 14239 | 44.8 | 317.6 | 84.6 | 14.8 |
| Component P3c | 11528 | 6.5 | 1779.0 | 68.6 | 82.9 |
| Component P3b | 1920 | 3.2 | 604.0 | 11.4 | 28.2 |

The specific HA activity of garlic lectins increased from 21.5 units/mg in crude extract to 1779 (ASA I) or 604 (ASA II) units/mg in the final step of purification (components P3c and P3b, respectively, of the second chromatographic step). The yield of garlic lectins are approximately 6.5 and 3.2 mg, in the case of ASA I and ASA II, respectively, from 100 g garlic bulbs.

### 5.4.5. Hemagglutination activity and glycoprotein binding assay

The major proteins in garlic are the low molecular weight mannose-binding agglutinins, and alliinase. The glycoprotein binding ability and hemagglutination activity of purified garlic lectins are summarized in Table 5.3. Purified ASA I and II show high binding for HRP and avidin compared to the crude extracts, and their binding ability is

comparable to that of the known mannose/glucose-specific lectin Con A. ASA II shows one-third the specific hemagglutination activity of ASA I.

**Table. 5.3. Glycoprotein-binding assay and HA activity of purified (ASA I and ASA II) garlic lectins**

| Protein* | Glycoprotein binding assay | | HA activity (units/mg) |
|---|---|---|---|
| | HRP (20 μg) ($A_{492}$) | Avidin-AP (1:2000) ($A_{405}$) | |
| Non-lectin (BSA) | 0.013 | 0.020 | No HA |
| Lectin (Con A) | 0.474 | 0.526 | 2132 |
| P3c (ASA I) | 0.395 | 0.489 | 1779 |
| P3b (ASA II) | 0.263 | 0.344 | 604 |

*Amount of protein coated: 10 μg

**5.4.6. SPT of purified garlic lectins on atopic and non-atopic subjects**

Skin prick test results with ASA I and II on atopic and non-atopic subjects are shown in Table 5.4.

.

Table 5.4. SPT results of ASA I and ASA II on human atopic and non-atopic subjects

| Protein | No. of subjects tested (n) | No. of subjects +ve by SPT | Percent positive | Wheal/ flare diameter (mm)* |
|---|---|---|---|---|
| ASA I | | | | |
| Non-atopic | 25 | 00 | 00 | 0-1/0 |
| Atopic | 45 | 12 | 26.6 | 3.5-4/5-10 |
| ASA II | | | | |
| Non-atopic | 25 | 00 | 00 | 0-1/0 |
| Atopic | 45 | 07 | 15.5 | 3-3.5/5 |

*Positive control: histamine base (1 mg/mL); 5-6/20-25
*Negative control: glycerinated PBS; 0-1/0

SPT on 45 atopic subjects (who had symptoms of any allergic disease) using ASA I and ASA II at 100 μg/ml, was found to be positive in 12 (26.6%) and 7 (15.5%) subjects, respectively; among 25 non-atopic subjects (healthy individuals without any symptoms of allergy) tested, none were found to be positive

**5.4.7. Histamine release (HR) ability of garlic agglutinins from human leukocytes**

In the case of atopic (n=3) and non-atopic subjects (n = 3), the HR assay was performed with ASA I and ASA II in the concentration range of 0.001 to 20 µg per mL, and, also the positive/negative control proteins. The positive reference control, con A, was found to induce HR in the range of 0.01 to 20 µg/mL, and maximum release was observed at ~1 µg/mL (data not shown), whereas non-lectin control, OVA, does not show any HR.

Both ASA I and ASA II were found to induce histamine release (HR) from leukocytes of atopic and non-atopic subjects (Fig 5.8); maximal release was seen at 1 μg/mL concentration. .

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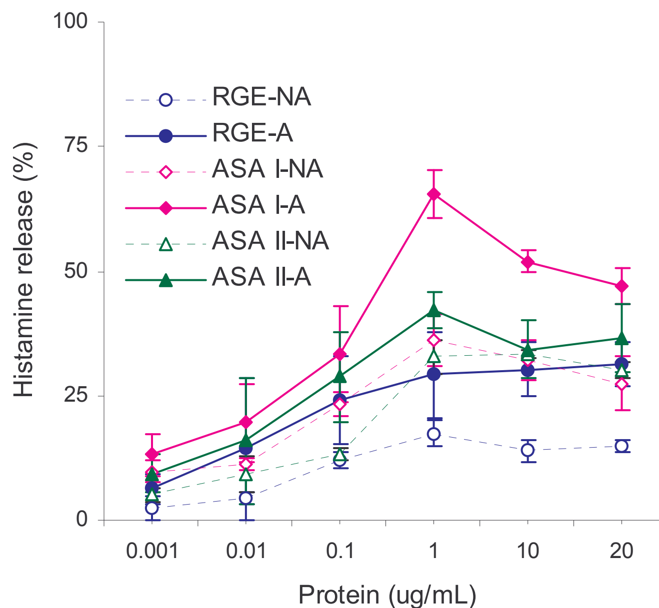



*Fig. 5.8. Histamine release from leukocytes of atopic (n=3) and non-atopic (n=3) subjects as a function of ASA I, ASA II and raw garlic extract (RGE) concentration (0.001 to 20 μg/mL). A: atopic subjects, NA: non-atopic subjects.*

ASA I is more potent compared to ASA II, and HR by ASA I in the case of the atopic subjects (61%) is significantly higher compared to that of the non-atopic subjects (36%). ASA II (HR for atopic 32%, non-atopic 26%) and raw garlic extract (HR for atopic 16%, non-atopic 23%) do not show a significant difference in HR between atopic and non-atopic subjects (Fig. 5.9). ASA I shows an effect similar to that of the reference lectin Con A in degranulating basophils present in leukocytes to release histamine

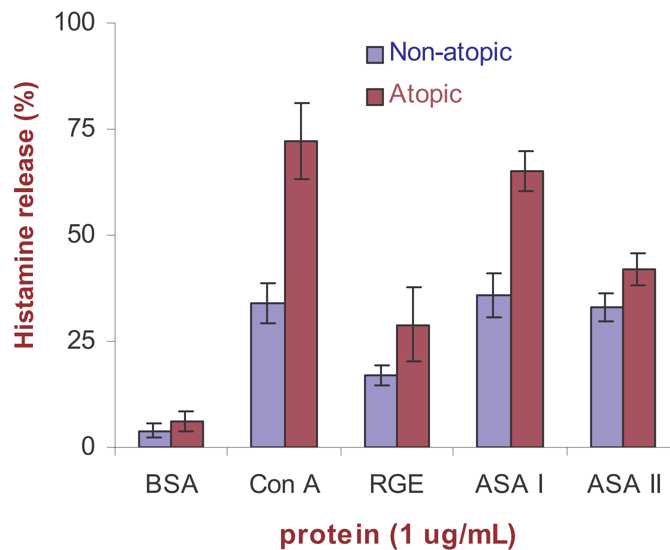



*Fig. 5.9. Comparison of HR from atopic and non-atopic subjects as a function of raw garlic extract (RGE) or garlic proteins at 1 μg/mL concentration. BSA: negative control; con A: positive control. Percent HR is taken from the data presented in Fig. 5.8.*

**5.4.8. Inhibition of histamine release by sugars**

Since the specificity of garlic lectins is for mannose, the inhibition of HR from leukocytes of an atopic subject was studied (produced at 1 μg/mL garlic lectins) in the presence of 50 μg/mL mannose or glucose (Fig. 5.10). There is a remarkable inhibition of HR by both ASA I and ASA II by mannose, and the inhibition is significant. On the other hand, glucose shows very weak inhibition of HR by ASA I and ASA II and appears to be not significant. However, both mannose and glucose show significant inhibition in HR induced by con A.

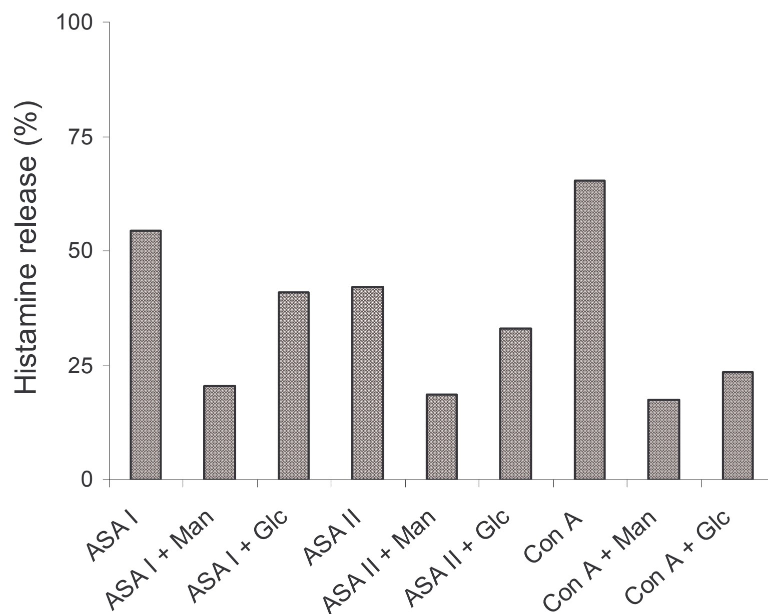



*Fig. 5.10. HR from leukocytes of an atopic subject at 1 μg/mL garlic lectins or con A, and its inhibition by mannose (Man), or glucose (Glc) at 50 μg/mL.*

### 5.4.8. HR from rat peritoneal exudate cells (PECs)

Histamine release was also observed with rat peritoneal exudate cells (PECs) using ASA I (57%), ASA II (26%) and garlic extract (17%). Mast cells constitute 15-20% of peritoneal exudate cells (Fig 5.11). .

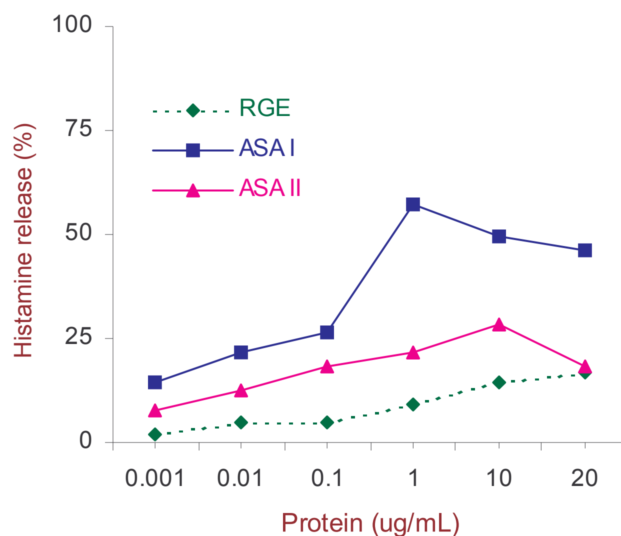
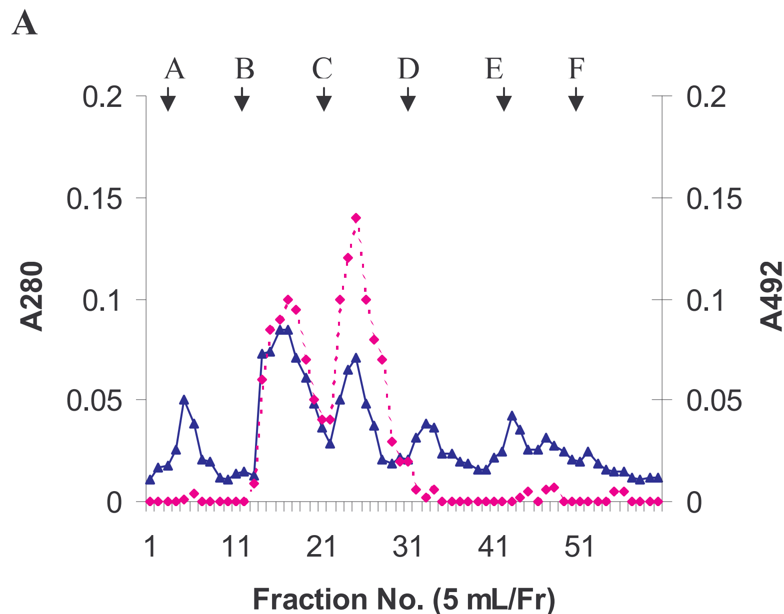



*Fig. 5.11. HR from rat peritoneal exudate cells by garlic lectins (ASA I, ASA II) and raw garlic extract (RGE) at 0.001 to 20 μg/mL protein concentration.*

It appears that garlic agglutinins are able to induce non-specific activation of mast cells by binding to the glycans of surface-bound IgE. Only ASA I shows an effect similar to that of the reference lectin Con A in degranulating mast cells

**5.4.9. Isolation of garlic lectins from raw and heat-processed garlic**

The isolation of garlic lectins from heat-processed garlic bulbs was relatively unsuccessful following the procedure identical to that described for the isolation of lectins from raw garlic extract in section 5.3.10. Clear raw and heat-processed extracts were subjected directly to SP-Sepharose chromatography for the elution of lectins. Gel permeation chromatography (Sephadex G-50) was not used in this purification. There is a significant loss in the total protein although heat-processed garlic extract retained a considerable amount of lectin which was confirmed by HA and HRP binding assays. The extract prepared from HPG was very viscous which did not flow through the column easily; instead, it formed a layer over the top of column material (SP-Sepharose). The SP-Sepharose profile of both raw and heat-processed garlic extracts are shown in Fig 5.12.



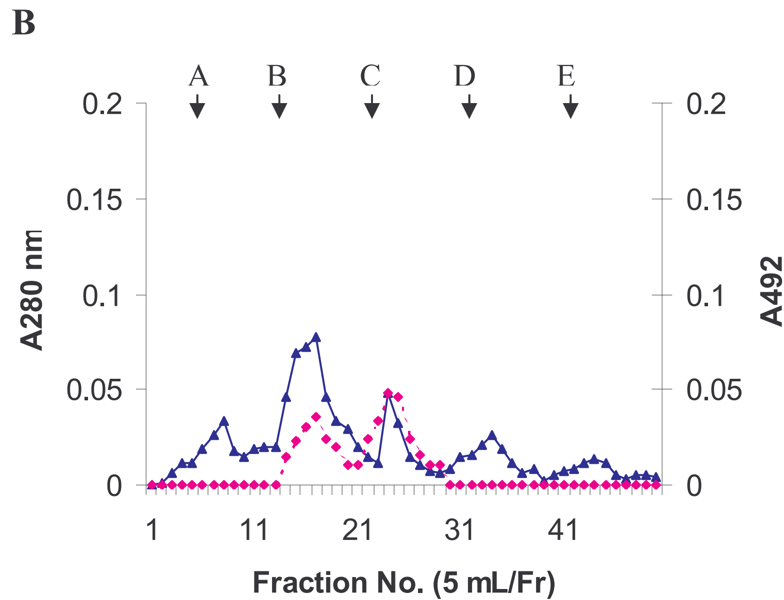



*Fig. 5.12. Isolation of garlic lectins from (A) raw garlic extract, RGE and (B) heat-processed garlic extract, HPGE on SP-Sepharose. Elution: step-wise using different concentrations of NaCl in 50 mM sodium acetate buffer, pH 4. Arrows indicate the point of application of buffer containing the indicated NaCl concentration (M): A, 0.1; B, 0.25; C, 0.5; D, 0.75; E, 1.0; F, 1.5. Protein detection: absorbance at 280 nm (-◆-). HRP-binding assay: absorbance at 492 nm (-◆-).*

## 5.5. DISCUSSION

Lectins are often the major proteins in some dietary sources. In some food sources, they represent the major storage proteins as found in majority of the legumes (Smeets et al., 1997). With the exception of the large group of Gramineae lectins, most interest in plant lectins focused on lectins in dicotyledonous plant species. However, evidence has accumulated that lectins are not confined to a single family of monocotyledonous species, but rather are widespread in this group. Moreover, the isolation and characterization of lectins from different species of the group revealed that some of them exhibit several interesting properties (Van Damme et al., 1991). For instance, the lectins from Galanthus, Narcissus, and Leucojum exclusively recognize mannose (Shibuya et al., 1988; Van Damme et al., 1987), which differs from all previously, reported mannose-binding lectins from the legume species that also bind Glc and GlcNAc. Studies within the Amaryllidaceae mannose-specific lectins occurring in

bulbs of species belonging to three genera Galanthus, Narcissus, and Leucojum (Van Damme et al., 1987), and it became evident that lectins similar to Amaryllidaceae also occur in Allieae of Alliaceae. Furthermore, Alliaceae and Amaryllidaceae lectins have similar molecular structure, agglutination properties and amino acid compositions. However, none of the lectins appear to be glycosylated (Van Damme et al., 1991). When the lectins from different Alliaceae species were compared, it is surprising that *A. ursinum* (ramsons), *A. vineale* (wild garlic), *A. sativum* (garlic) and *A. moly* (golden garlic) lectins differ from *A. cepa* (onion) and *A. porrum* (leek) lectins in that they are proteins comprised of two subunits. In all Amaryllidaceae and Alliaceae lectins represent one of the most predominant proteins, quantitatively, in crude bulb extracts (Van Damme, et al., 1987, 1991).

Most of the legume lectins have been well studied. Concanavalin A (Con A) from jack bean (*Canavania ensiformis*) has been investigated extensively in terms of the protein structure, saccharide specificity (Pazur et al., 2000; Zhao et al., 2002), and its interaction with a variety of cells (Margo, 1977; Sullivan et al., 1975). It has been shown to interact with basophils and mast cells specifically by binding to the carbohydrates on the Fc portion of cell-bound IgE, resulting in the release of histamine and other biological mediators. IgE contains 10-12% carbohydrates (Arnold et al., 2004) as compared to only 3-4% carbohydrates in the case of IgG. Although many other lectins have been isolated and characterized from plant food sources, studies of their interaction with cells of the immune system have not been done in detail and their role in non-allergic food hypersensitivity is limited.

Garlic is known as a potent spice and consumed world wide as an ingredient in a variety of food preparations. Two major proteins, alliinase and garlic lectins are known to constitute approximately 96% of the total proteins in garlic (Wen et al., 1995). Garlic lectins alone in the garlic bulbs constitute approximately 50% of the total protein. They are the major storage proteins in garlic bulbs and are known to be expressed more during the bulb development and found to be at maximal levels in the developed young bulbs as compared to the dried old bulbs (Smeets et al., 1997a; 1997b). The present

study was undertaken to investigate the effect of dietary mannose-specific lectins on mast cells and basophils of non-atopic and atopic subjects; garlic lectins were choosen as the prototype. This class of lectins includes the monocot mannose-specific lectins, which have maximum specificity for terminal or core mannosyl residues. The most well studied lectin in this category based on the sugar specificity is the Concanavalin A (Con A) from jack bean, which is from a non-dietary source. Con A (Zhao et al., 2002; Margo, 1977) that has specificity to man/glc is used as a model reference lectin.

This chapter describes the purification of garlic lectins (ASA I and ASA II) from garlic bulbs using two steps, namely, gel filtration and cation-exchange chromatography. The glycoprotein-binding ability of purified lectins and their hemagglutination activities were assessed to relate their role in the interaction of these lectins with immune cells involved in hypersensitivity. The garlic agglutinins have been purified from raw garlic extract using a combination of gel filtration on Sephadex G-50 and cation-exchange chromatography on SP-Sepharose at pH 4. Using step-wise elution from SP-Sepharose, ASA II eluted at 250 mM NaCl, and ASA I at 500 mM NaCl concentration. Heat-processed garlic (garlic cooked in water for 15 min) extract showed one-third decrease in specific hemagglutination activity, and the protein content was low after extraction (7.2%). Dried garlic powder extract showed a similar specific hemagglutination activity and glycoprotein binding, as those of raw garlic extract. Purified ASA I and II show high binding for the glycoproteins (HRP and avidin-alkaline phosphatase) compared to the crude extracts, and their binding ability is comparable to that of the prototype mannose/glucose-specific lectin, concanavalin A (Con A). ASA II exhibited one-third the specific hemagglutination activity of ASA I. There is also a report that a high mol. wt. agglutinin ($ASA_{110}$ from *Allium sativum*) is specific to D-mannose at higher concentration, its specificity is more towards mannan linkages (Gupta and Sandhu, 1997). This report suggests that $ASA_{110}$ belongs to phylogenetically related group of proteins which recognize complex carbohydrate structures (Kaku et al., 1988). Affinity of $ASA_{25}$ and $ASA_{110}$ for a glycoprotein was assesed using asialofetuin and may

possibly be attributed more to $\alpha(1\rightarrow3)$ than $\alpha(1\rightarrow4)$ or $\alpha(1\rightarrow6)$ mannan linkages in the case of $ASA_{110}$ (Gupta and Sandhu, 1997).

SPT of atopic subjects using garlic lectins revealed that ~26% (in the case of ASA I) and 15% (in the case of ASA II) of atopic subjects showed a positive reaction, which translates to ~5.2% for ASA I and 3% for ASA II, in the general population. The SPT results are unusually high for a food protein compared to the incidence of 3-4% for food allergy in adults (Prussin and Metcalfe, 2003; Kjaer and Frokiaer, 2005). This may be due to the non-specific interaction of mannose-specific lectins with cell-bound IgE of mast cells. Since cell-bound IgE is a glycoprotein rich in oligosaccharides (~12%) in its Fc portion (both oligomannose and complex bi-antennary types), the composition and structures of the *N*-linked glycans on the heavy chain of IgE (Baenziger and Kornfeld, 1974) was examined. Among the glycans of human IgE, ~86% of glycans terminate in galactose or sialic acid (Arnold et al., 2004), which represent complex bi-antennary type glycans (Baenziger and Kornfeld, 1974). The total IgE level was 2 to 8-fold higher in atopic subjects (who are positive to garlic lectins by SPT) as compared to non-atopics; garlic lectins can interact with and activate these mast cells. Based on the criteria presented in Chapter 3, Table 3.1, atopic and non-atopic subjects were selected for HR studies using garlic lectins.

Both ASA I and ASA II were found to induce histamine release (HR) from leukocytes of atopic and non-atopic subjects; maximal release was seen at 1 $\mu$g/mL concentration. ASA I is more potent compared to ASA II in inducing HR, and HR by ASA I in the case of the atopic subjects is significantly higher (2-fold) compared to that of the non-atopic subjects. ASA II and raw garlic extract do not show a significant difference between non-atopic and atopic subjects. Similar results were found with rat PECs. It appears that garlic agglutinins are able to induce non-specific activation of basophils and mast cells by binding to the glycans of surface-bound IgE. The SPT and HR results from non-atopic and atopic subjects clearly indicate that garlic agglutinins activate and degranulate mast cells and basophils by interacting with the trimannosidic core of IgE glycans (Fig 4.11). It seems likely that a higher density of IgE on mast

cells/basophils of some atopic subjects, and/or individual differences in the glycans (on the cell surface or on IgE molecules) could account for susceptibility of some individuals to a dietary lectin.

### 5.6. SUMMARY AND CONCLUSIONS

The major proteins in garlic bulbs are alliinase and the low mol. wt. mannose-specific agglutinins or lectins ($ASA_{25}$). Purified ASA I and II show high binding for the glycoproteins (HRP and avidin-alkaline phosphatase) compared to the crude extracts, ASA II exhibited one-third the specific hemagglutination activity of ASA I. Skin prick test (SPT) on 45 atopic subjects was found to be positive in 12 (26.6%) and 7 (15.5%) subjects for ASA I and II, respectively; none of non-atopics was found to be positive. Both ASA I and ASA II were found to induce histamine release (HR) from leukocytes of atopic and non-atopic subjects; HR by ASA I in the case of the atopic subject (61%) is significantly higher compared to that of the non-atopic subject (36%). ASA II and raw garlic extract do not show a significant difference in HR. The results of SPT and HR clearly indicate that garlic agglutinins activate and degranulate mast cells and basophils by interacting with the trimannosidic core of IgE glycans. It seems likely that a higher density of IgE on mast cells/basophils of some atopic subjects, and/or individual differences in the glycans (on the cell surface or on IgE molecules) could account for susceptibility of some individuals to a particular dietary lectin.

# Chapter 6

# Modulatory effects of potato and garlic lectins in relation to mitogenicity

## 6.1. INTRODUCTION

Molecules, cells and organisms display information about themselves in the form of glycoconjugates. This information is decoded by highly specific carbohydrate-binding proteins called lectins. As a consequence, lectins have found widespread application in probing the architecture and dynamics of cell surface carbohydrates during cell division, differentiation, and malignancies as well as in the isolation and characterization of glycoconjugates (Liener et al., 1986; Sharon and Lis, 1989).

Lectins are a structurally very diverse class of proteins that bind carbohydrates with considerable specificity but moderate affinity (Lis and Sharon, 1998). Lectin-carbohydrate interactions are involved in a wide variety of biological functions, including recognition (Naeem et al., 2001), agglutination (Khan et al., 2002), adhesion, cancer metastasis, bacterial and viral infections, inflammation and cellular growth (Varki, 1993). Lectins have captured the attention of a large number of researchers on account of the various exploitable activities that they exhibit, including their proliferative effects on various cell types. Recognition of cell-surface carbohydrates by lectins has broad implications in important biological processes. The ability of lectins to detect subtle variations in carbohydrate structures on the surface of cells and tissues has made them a paradigm for protein-carbohydrate recognition (Sharon and Lis, 2004). Lectins display a considerable repertoire of carbohydrate specificities. These characteristics, together with the ability to stimulate lymphocytes as well as other cells, have made lectins an important diagnostic and experimental tool to study the various aspects of cell growth and differentiation, taking lymphocytes as the model cell type (Kilpatrick et al., 1990).

The discovery of lectin-mediated mitogenesis by Nowell (1960) stimulated interest in the properties of lectins while advancing knowledge of immunology in the sixties. Although some lectins are polyclonal activators both in vitro and in vivo (Barker, 1969) others may display a broad range of activities towards human lymphocytes (Kilpatrick, 1999). Certain lectins are mitogenic (Ling, 1968), in that they can stimulate the transformation of cells from the resting phase to blast-like cells, which may subsequently undergo mitotic division (Barker, 1969; Kilpatrick, 1999). The discovery

that certain lectins are potent mitogens has opened up a new arena for scientists to study the probable role of lectins in cell growth and development. One of the most useful results of this proliferative ability of lectins has been increased understanding of the relationship between chromosomal abnormality and human diseases, which has tremendously helped the diagnosis (Kilpatrick, 1991; 1995). Besides other cells, lymphocytes have been the usual target cells for mitogenic assays, and the study of lectin-lymphocyte interactions has made substantial contribution to elucidating the mechanism of lymphocyte activation and its control, thereby contributing to the current understanding of cell growth and development (Kilpatrick, 1999).

Plant lectins have been shown to modulate positively or negatively the steps of the cell growth-regulating pathway. Certain lectins inhibit cell growth, such as the trypsin-stable lectin from *Vigna sesquipedalis* (Jack and Wong, 2003), wheat germ agglutinin, and *Viscum album* (mistletoe) agglutinin (Hideki et al., 2003; Miyoshi et al., 2001), while others stimulate it. Lectins do not stimulate lymphocytes in a preferential manner; they bind to carbohydrate receptors on the surface of all those lymphocytes, which contain the same sugar moieties appropriate for the specificity of the mitogenic lectins. It has been observed that over 80% of the total lymphocytes may be induced on stimulation with the same lectin under appropriate conditions (Hume and Weidaman, 1980). Lectins such as phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM) have been extensively used to study lymphocyte function, *in vitro*, for many years (Miller, 1983). These proteins were the first recognized polyclonal activators of T-cells. They bind to a number of glycoproteins expressed on the plasma membrane of T-cells (Dam et al., 2000). Con A and PHA (Wang et al., 2001), have been found to be selective T-cell mitogens when compared to their effect on B-cells, whereas PWM and LPS are B-cell mitogens (Ashraf and Khan, 2003). Thus, most lectins stimulate only T-cells and are inhibitory for the mitosis of the other class of lymphocytes.

The prerequisite in mitogenic activities of lectins seems to be initiated by binding of lectins to cell surface carbohydrates (Axelsson et al., 1978; Chilson and Chilson,

1989), which constitute a component part of lectin-specific receptors. Lectin-induced membrane stimulation reactions lead to the generation of second messengers such as diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($IP_3$), leading to an increase in cytosolic $Ca^{2+}$ concentration (Yakura, 1994; Edgar et al., 1998). It also induces the synthesis of specific proteins not found in resting lymphocytes. Soon after activation, lymphocytes release a variety of biologically active polypeptides known as lymphokines (Cohen and Oppenheim, 1979), the best characterized of these is interleukin 2 (IL-2). IL-2 production and expression of its receptor (Fig 6.1), IL-2R (Brown et al., 1989; Nel, 2002), has been thoroughly studied (Kawakami et al., 1988).

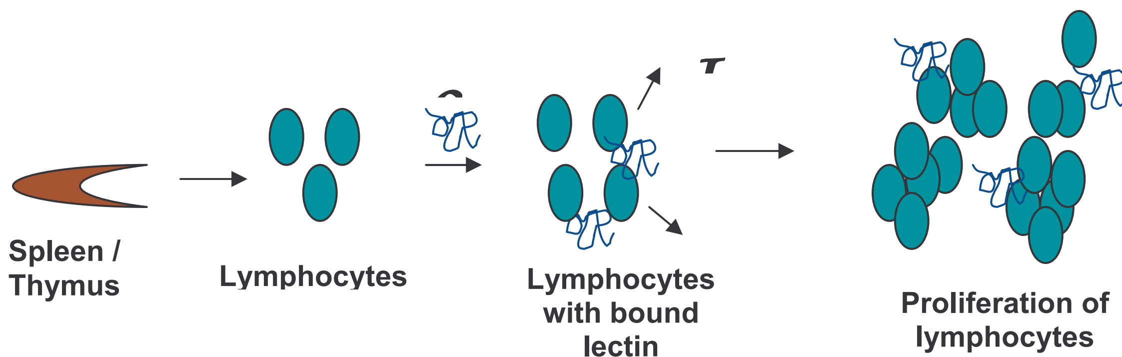



*Fig. 6.1. Lectin stimulated IL-2 production from splenocytes and thymocytes. Binding of lectin to glycoproteins on lymphocytes induces variety of signals resulting in mitogenic, co-mitogenic or anti-mitogenic effects.*

Studies on wheat germ agglutinin (Novogrodsky and Katchatski, 1973; Greene et al., 1981) have lead to the proposition of a new model of mitogenic stimulation by lectins. According to this model, positive and negative modulation of lymphocyte function is exercised *via* discrete stimulatory and inhibitory domains on the lymphocyte membrane (Fig 6.1); mitogenic lectins bind to receptors that are distributed in the stimulatory regions or domains (Marth et al., 1989), whereas non-mitogenic or anti-mitogenic lectins bind to receptors that are confined to the inhibitory regions or domains (Kilpatrick and McCurrach, 1987). The molecular events mediating the lymphocyte response to lectins are incompletely understood, although much evidence supports the hypothesis that both the mitogenic and pro-apoptotic effects of the agents involving the biochemical cascade initiated by the CD3/T-cell antigen receptor (TCR complex), have been shown to have

role in cell response to cytokines and growth factors, thus indicating the probable role in lymphocyte proliferation by mitogenic lectins (Pani et al., 2000; Nel, 2002).

The mitogenic effect of garlic extracts as a whole (Colic and Savic, 2000; Colic et al., 2002) and the high molecular weight glycoprotein agglutinin from garlic designated $ASA_{110}$ (Gupta and Sandhu, 1996), has been studied on T-lymphocytes. Many lectins such as con A, PHA, WGA and PWM bind to specific carbohydrate residues on the lymphocyte membrane and induce a cascade of events leading to cell activation, including proliferation, production of lymphokines and differentiation (Gupta and Sandhu, 1996; Miller 1983). Hence, it will be interesting to study the mitogenic potential of dietary lectins, as they cause *in vivo* modulation of lymphoid tissues. This chapter describes the modulatory effects of the mannose-specific garlic lectins (ASA I and ASA II) and oligo-GlcNAc specific potato lectin on lymphocytes from human peripheral blood and murine splenocytes and thymocytes, in relation to mitogenicity.

## 6.2. MATERIALS

This study was undertaken after clearance by the Institutional Human as well as Animal Ethics Committees; informed consent was obtained from all human volunteers for obtaining peripheral venous blood in the age range of 18-60 years.

Potato lectin and garlic lectins were purified in the laboratory as described earlier in Chapter 3 and 5, respectively. Spleen and thymus were obtained from BALB/c mice procured from Central Animal House Facility, Indian Institute of Science, Bangalore, India. Concanavalin A (Con A), phytohemagglutinin (PHA), Ficoll-hypaque, RPMI-1640 medium were products of Sigma-Aldrich Co., St. Louis, MO, USA. Tissue culture grade sodium bicarbonate, L-glutamine, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide A.R.], and NBT (Nitroblue tetrazolium A.R.) were purchased from HiMedia Laboratories Ltd., Mumbai, India. Fetal calf serum (FCS) was obtained from Sera-lab (Sussex, England). Tissue culture plates were products of Costar Ltd., Cambridge, MA, USA. All other chemicals and reagents were of analytical grade.

## 6.3. METHODS

### 6.3.1. Raw and heat-processed potato/garlic extracts and their lectins

Raw and heat-processed potato/garlic extracts (50% w/v) were prepared using phosphate buffered saline (PBS) following the procedures described in the previous chapters (§ 3.3.8 of chapter 3 and § 5.3.1 of chapter 5). Raw and heat-processed extracts were analyzed for protein content by dye-binding assay (Bradford, 1976).

Potato lectin (STA) and garlic lectins (ASA I and ASA II) were purified to homogeneity following the chromatographic procedures as outlined in the previous chapters; they were used at a protein concentration of 1 mg/mL. Similarly, Con A and PHA were also prepared at 1 mg/mL concentration using PBS.

### 6.2.2. Preparation of complete RPMI-1640 media for proliferation assay

RPMI-1640 cell culture medium was used for all experiments. For incomplete medium, powdered medium was added to triple distilled filtered water, and dissolved by gentle stirring. The pH of the medium was lowered to 4.0 with 1 N HCl in order to completely dissolve the medium; after it has dissolved, the pH was raised back to 7.2 with 1 N NaOH. Later, tissue culture grade sodium bicarbonate was added to a strength of 7.5%, the final volume was made with water, and the pH adjusted to 7.2.

Incomplete medium was supplemented with 10% v/v fetal calf serum, 1% sodium pyruvate, 2 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL of streptomycin to obtain a complete medium, which was used for proliferation assay (Mosmann, 1983).

### 6.3.3. Isolation of human peripheral blood lymphocytes (PBLs)

Ten milliliters of venous blood were drawn from healthy normal subjects using a disposable syringe. Five milliliters of each sample was transferred to HiAnticlot vials (heparin-coated flat-bottom polystyrene vials with polypropylene caps from HiMedia Laboratories Ltd., Mumbai, India). The contents of the vials were swirled slowly to avoid coagulation. After a few minutes, the heparinized blood was layered carefully on Ficoll-hypaque (density = 1.077 g/mL) contained in a 15 mL-graduated polystyrene tube. The

tubes were kept at 25ºC for 90 min to allow the separation of erythrocytes, lymphocytes and plasma based on their density. Next, the tubes were centrifuged at 250 × g at 25ºC for 20 min. Centrifugation at a lower temperature (4°C) was avoided since this result in cell clumping and poor recovery (Colic and Savic, 2000). After centrifugation, below the plasma layer, a circle of white translucent coat containing lymphocytes was aspirated carefully using a Pasteur pipette. The cells were then resuspended in isotonic phosphate-buffered saline (PBS) and mixed by gentle aspiration. The buffy coat containing lymphocytes was washed 4-5 times using PBS at 4°C, and finally were placed in complete RPMI-1640 medium.

### 6.3.4. Isolation of murine splenocytes and thymocytes

Spleen and thymus were collected under aseptic conditions from normal BALB/c mice (23-25 g, 12-weeks-old) after sacrification, placed in isotonic phosphate buffered saline (PBS) (Colic et al., 2002). These tissues were separately minced using a pair of scissors and passed through a fine steel mesh to obtain a homogenous cell suspension. The cells were pelleted to remove the tissue debris. After centrifugation (380 x g at 4°C for 10 min), the pelleted cells were washed three times with PBS (400 x g at 4°C for 10 min) and finally resuspended in complete RPMI-1640 medium.

### 6.3.5. Removal of contaminating erythrocytes from the cell suspensions

The pellet obtained after washing with PBS was resuspended in a modified ammonium chloride buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, pH 7.4 containing 10 mM sodium edetate) and incubated at 4°C for 5 min to remove the erythrocytes (Boyam, 1968). After the cells were centrifuged at 400 × g at 4°C for 20 min, the pellet was mixed in physiological salt solution (PSS) [137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.6 mM $CaCl_2$, 10 mM HEPES, 0.05% gelatin and 6.45 mM $NaH_2PO_4$ at pH 7.4], washed three times in the same buffer, and finally resuspended in RPMI-1640 medium.

### 6.3.6. Counting of lymphocytes and determination of viability

The isolated lymphocytes from normal human subjects and mice spleen and thymus were counted using crystal violet stain. An aliquot (5 μL) of cell suspension was taken and diluted with 250 μL of diluent buffer (PBS with 1% BSA) to which 10 μL of crystal violet stain (stock) was added. The mixture was kept at room temperature for a minute, and then charged to hemocytometer using a clean fine pipette tip. The leukocytes were observed under 10X low power eyepiece and counted in the outer four chambers of the hemocytometer. The cell concentration was adjusted to 2.5 x $10^6$ cells/mL and used for proliferation assay.

Percentage viability of lymphocytes in the isolated cell suspension was checked by Trypan blue exclusion method. For the cell viability determination, an aliquot of cell suspension was taken and mixed with 0.2% Trypan blue at 1:1 dilution and kept at 25°C for ~2 min. The cell suspension with Trypan blue was charged to hemocytometer and then observed under microscope. Cells, which are dead or partially damaged, appear as dark blue against a light blue background, since they take up the dye. The viable cells appear clear without any stain against the light blue background.

### 6.3.7. MTT assay for cellular proliferation

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay is a simple colorimetric method to measure cytotoxicity, proliferation, or cell viability (Mosmann, 1983). MTT is a yellow, water-soluble, tetrazolium salt. Metabolically active cells are able to convert this dye into a water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring (Mosmann, 1983). Formazan crystals, was dissolved and quantitated by measuring the absorbance of the solution at 570 nm.

The assay was performed in tissue culture plates (96 wells microtiter plates); the wells were filled with complete medium followed by lectins (0.01 to 10 μg/mL) and lymphocytes (100 μL of 2.5 x $10^6$ cells/mL). Plates were incubated in a $CO_2$ incubator at 37°C with 5% $CO_2$ for 72 h. Lymphocytes in the absence of lectins represent control, and blank was carried out with complete medium only. After incubation for 72 h, 5 μL of

5 mg/mL MTT solution (MTT was dissolved in 0.1 M Tris-buffered saline, which was then filtered to remove any insoluble residues) was added and incubated for further 4 h under the same conditions. After removing the plates, the samples were aspirated to an ependorf tube, and centrifuged at 750 x g at 4°C for 15 min. Supernatant was removed and the blue formazan crystals were resolubilized in 500 μL of isopropanol with 0.04 N HCl under agitation. After dissolving the crystals, 100 μL of each sample were taken in microtiter plates. Plates were read in a microplate reader at 570 nm.

**6.3.8. Nitric oxide (NO) assay**

NO is a gaseous free-radical molecule which is catalytically generated by cellular nitric oxide synthase (NOS) upon conversion of L-arginine to L-citrulline. The amount of NO produced in the medium (incubated with human PBLs and lectins) was determined by assaying (Morihara et al., 2002) its stable end product, $NO_3^-$ (nitrate). Briefly, equal volumes (100 μL) of sample and Greiss reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine in 5% $H_3PO_4$) were mixed in a 96-well microtiter plate at room temperature. The absorbance was then measured at 540 nm in a microplate reader. A range of sodium nitrate dilutions served to generate a standard curve for each assay.

**6.3.9. Nitro blue tetrazolium (NBT) assay for reactive oxygen species**

The rat peritoneal exudate cells containing macrophages were isolated according to the procedure explained in chapter 4 using Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.4 mM $NaH_2PO_4$, and 5.6 mM glucose, pH 7.4) containing 0.1% BSA. The cells obtained were washed twice with Tyrode buffer by centrifugation at 200 × g at 4°C for 20 min.

For the NBT assay (Aukrust et al., 1994), peritoneal cells (1 x $10^5$ cells/mL) were taken in a volume of 100 μL, 400 μL of Hank's balanced salt solution (HBSS) (800 mg NaCl, 40 mg KCl, 6 mg $KH_2PO_4$, 4.8 mg glucose, 9.8 mg $Na_2HPO_4$, 14 mg $CaCl_2$ in a final volume of 100 mL) containing lectins (0.1, 1, 10 μg/mL) was added with 0.4% BSA. The samples were preincubated at 37°C for 10 min, then 2 mM NBT (40 μL/each tube)

was added and further incubated at 37°C for 20 min. Reaction was arrested by the addition of 2.5 mL ice cold HBSS. After the tubes were centrifuged at 400 x g for 10 min, the supernatant was discarded and the blue formazan crystals were dissolved by adding 2 volume of dioxane. After centrifugation, absorbance of the supernatant was read at 540 nm.

## 6.4. RESULTS

### 6.4.1. Mitogenic effect of potato and garlic extracts on human PBLs

Raw and heat-processed potato extracts (RPE and HPPE) and garlic extracts (RGE and HPGE) were checked for their proliferative effects on human PBLs. Raw garlic extract (RGE) has mitogenic and co-mitogenic effect, whereas heat-processed garlic extract (HPGE) has half the mitogenic effect shown by RGE. The results are shown in Fig 6.2. In the case of co-mitogenic activity with Con A, RGE shows added stimulation whereas HPGE does not.

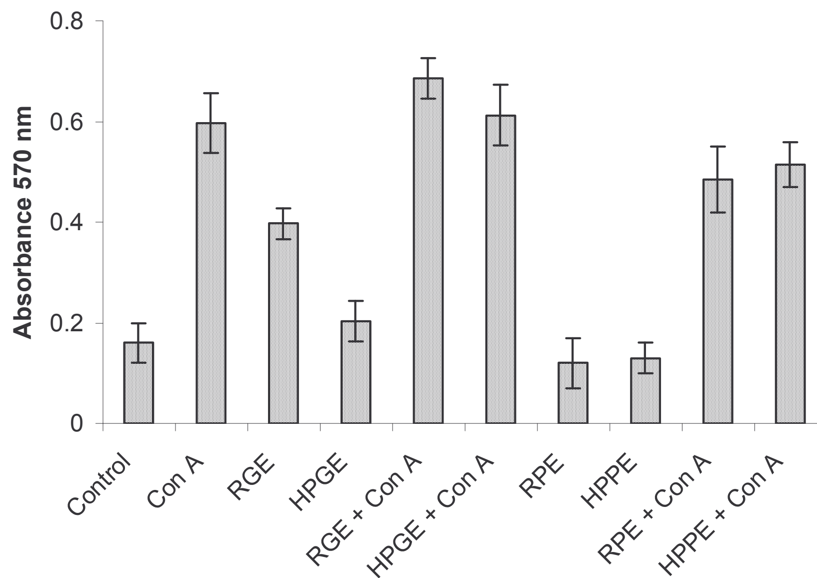



*Fig.6.2. Mitogenic stimulation of human PBLs by raw and heat-processed garlic and potato extracts. Con A was used as reference mitogen. Human PBLs were used at 2.5 x $10^5$ cells/mL. The extracts were used at a fixed protein concentration of 10 $\mu$g/mL. For mitogenic and co-mitogenic activity, all the extracts were used at protein concentration of 10 $\mu$g/mL, Con A was used at 5 $\mu$g/mL concentration.*

Con A was used as a reference mitogen; it induces a proliferation of 3.5 to 4 fold as compared to control cells, whereas RGE shows 2.5 fold, and HPGE shows only 1.25 fold. Potato extracts (RPE and HPPE) do not show mitogenic and co-mitogenic activity. When human PBLs were stimulated with RPE along with Con A, RPE was found to decrease, slightly the effect of Con A. HPPE does not have any modulatory effects on human PBLs (Fig 6.2). Both potato extracts were found to have no effect on human PBLs.

### 6.4.2. Mitogenic stimulation of human PBLs by purified garlic lectins (ASA I and ASA II) and potato lectin (STA)

The dietary lectins from garlic bulb (ASA I and ASA II) and potato tuber (STA) were purified to homogeneity as described in chapters 5 and 3, respectively. The purified lectins were tested for their ability to proliferate human PBLs. Initially, all the three lectins along with positive reference mitogen, Con A, were checked in the concentration range of 0.01 to 10 μg/mL concentration. The results are shown in Fig. 6.3.

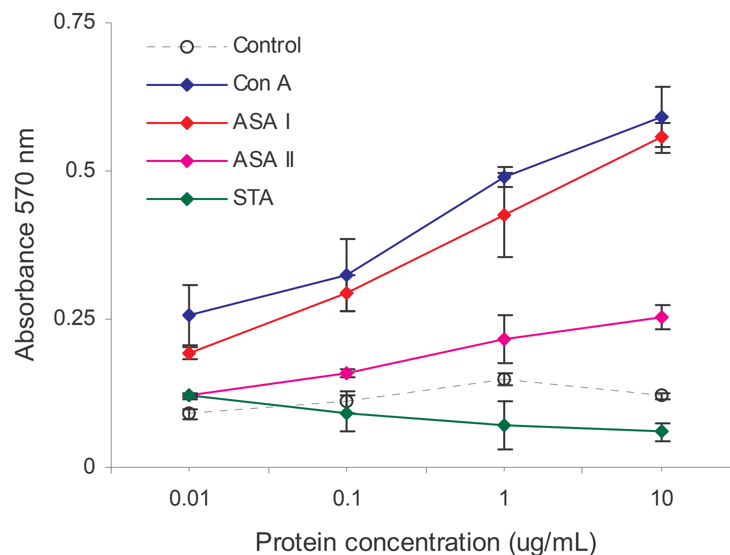



*Fig. 6.3. The immunomodulatory effects of garlic lectins (ASA I and ASA II) and potato lectin (STA) on human PBLs in the concentration range of 0.01 to 10 μg/mL. Con A was used as a reference positive for lymphocyte proliferation.*

Both garlic lectins ASA I and II show stimulatory effects with human PBLs whereas STA shows inhibitory effects at higher concentrations (1 and 10 μg/mL) and no effect at lower concentrations (0.01 to 0.1 μg/mL). ASA I behaves similar to Con A in its potential to cause proliferation of human PBLs (Fig 6.3). However, ASA II shows only a mild mitogenic effect as compared to control.

### 6.4.3. Mitogenic and co-mitogenic effect of purified lectins

Both garlic lectins, ASA I and ASA II, were found to be mitogenic and co-mitogenic for human PBLs, whereas STA was neither mitogenic by itself nor co-mitogenic when added with a known mitogen. The results are shown in Fig 6.4. ASA I shows a similar effect as that of the known mitogen Con A, and an increased response is seen in the presence of Con A (significant at $P \leq 0.001$). ASA II shows approximately half of the response seen for ASA I and Con A, but in the presence of con A, ASA II shows only a slight increase in cell proliferation (compared to Con A alone) which is not significant at $P \leq 0.05$. Potato lectin (STA) does not show any stimulatory effect; in the presence of con A, STA reduces the response induced by Con A by 30-35%, which indicates the inhibitory effect of potato lectin towards human PBLs.

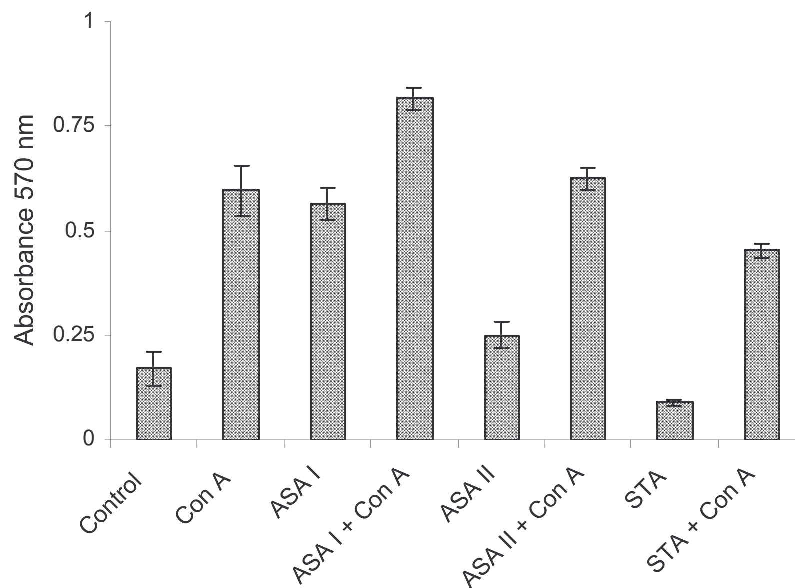



*Fig. 6.4. Mitogenic and co-mitogenic effects of garlic lectins and potato lectin on human PBLs. All the lectins were used at 5 μg/mL concentration. Cells used: $1 \times 10^5$ cells/mL.*

The proliferative effect of garlic lectins, potato lectin and garlic/potato extracts on human PBLs are shown in terms of proliferation index in Fig 6.5. The index for control (untreated cells) taken as 1.0, and for others are represented as fold increase or decrease over the control. Proliferation index is calculated by dividing the absorbance of test by absorbance of the control. ASA I and Con A shows 3.5 fold increase whereas ASA II shows only 1.5 fold, indicating that both ASA I and ASA II are mitogenic. On the other hand, STA showed a proliferation index of 0.65 and appears to be antimitogenic.

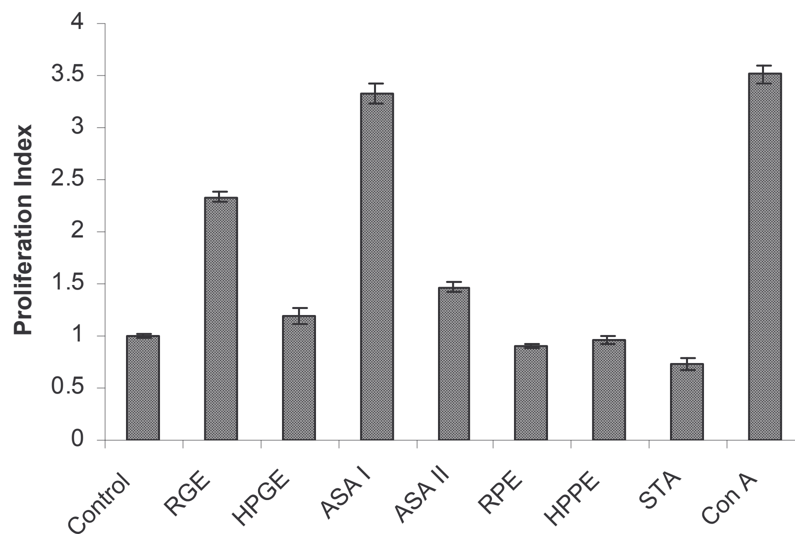



*Fig 6.5. Proliferation index of purified lectins and extracts from garlic and potato on human PBLs. The extracts were used at 10 $\mu$g/mL, purified lectins and Con A at 5 $\mu$g/mL concentration. Proliferation index was calculated by dividing absorbance of the test by absorbance of control.*

### 6.4.4. Modulatory effects of garlic lectins on murine splenocytes

Murine splenocytes were isolated from the spleen obtained from adult BALB/c mice. The splenocytes were tested for proliferation by garlic lectins ASA I and ASA II at 0.1 - 10 $\mu$g/mL concentration. Con A and PHA which are known T-cell mitogens were used as reference positive mitogens, and the cells in the absence of any lectin served as control. The result of splenocytes stimulation is shown in Fig 6.6. There is a significant difference (at $p \leq 0.001$) in the proliferation of splenocytes by ASA I at 1 and 10 $\mu$g/mL, and this effect is comparable to those of the reference mitogens. ASA II shows only a

slight increase in stimulatory effect and is not significant at $p \leq 0.001$ as compared to con A, but in comparison to control the effect is significant at $P \leq 0.05$.

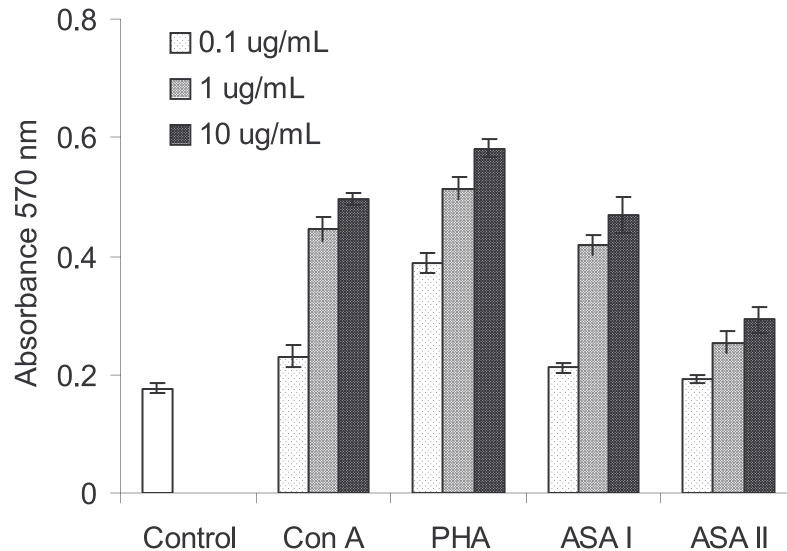



*Fig. 6.6. Immunostimulatory effects of garlic lectins ASA I and ASA II on murine splenocytes. The concentration range of lectins tested is 0.1 to 10 μg/mL. Con A and PHA are known T-cell mitogens, and represent reference positives.*

**6.4.5. Modulatory effects of garlic lectins on murine thymocytes**

Murine thymocytes were isolated form the thymus obtained from adult BALB/c mice. Thymocytes were stimulated by garlic lectins ASA I and ASA II at 0.1 - 10 μg/mL concentration. The results are shown in Fig 6.7. The stimulatory response of garlic lectins for murine thymocytes seems to be more as compared to human PBLs and murine splenocytes. Similar trend of activation by garlic lectins is seen as in the case of murine splenocytes. The effect of ASA I is comparable to that of Con A and PHA. However, ASA II shows a slightly higher degree of activation for thymocytes compared to splenocytes.

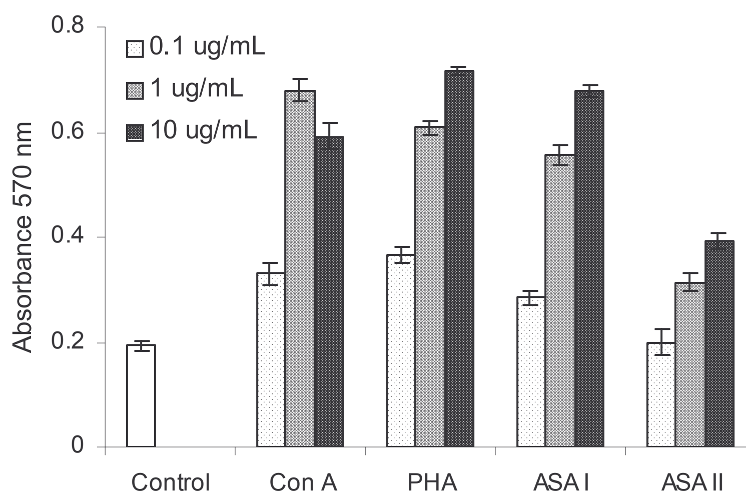



*Fig 6.7. Immunostimulatory effects of garlic lectins ASA I and ASA II on murine thymocytes. The concentration range of lectins tested is 0.1 to 10 μg/mL. Con A and PHA are known T-cell mitogens, and represent reference positives.*

### 6.4. 6. Comparison of the effect of garlic lectins on murine lymphocytes

Both the garlic lectins stimulate murine lymphocytes to varying degrees. The comparative results of the effect on these lectins on splenocytes and thymocytes are shown in Fig 6.8.

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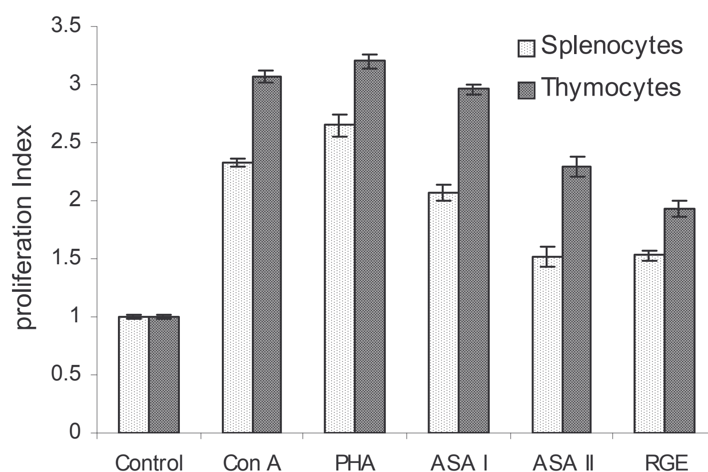


*Fig 6.8. Comparison of the proliferation index of garlic lectins on murine splenocytes and thymocytes. All lectins are used at 5 μg/mL concentration. Cell concentration used is 1 x $10^5$ cells/mL.*

ASA I show stronger stimulatory effect with both splenocytes and thymocytes. ASA II shows about 25-30% less effect compared to ASA I, but it shows considerably more response with thymocytes than splenocytes. Raw garlic extract (RGE) has significant modulatory effect on both splenocytes and thymocytes.

### 6.4.7. Effect of garlic lectins on nitric oxide (NO) production from human PBLs

The induction of nitric oxide production from human PBLs by garlic lectins was studied. A calibration curve was prepared using sodium nitrate in the concentration range of 0 -10 μM, and shows a linear correlation with $R^2$ of 0.0995 (Fig 6.9).

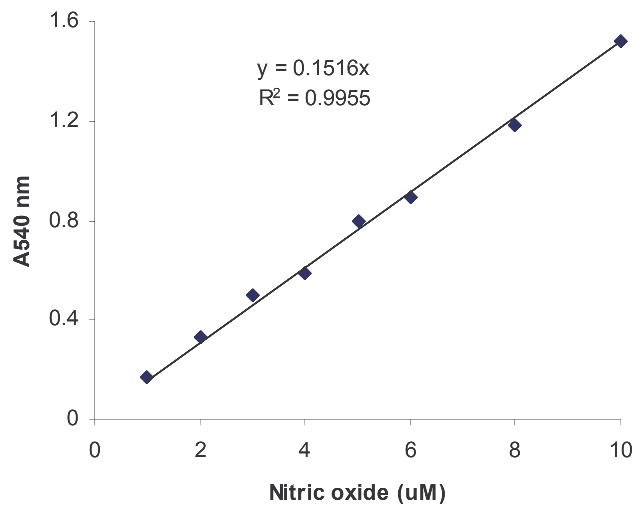


*Fig. 6.9. Calibration curve for nitric oxide (NO) in the concentration range of 0 to 10 μM. Volume range: 0-500 μL.*

Garlic extracts and purified garlic lectins do not show a significant increase in the NO production from human PBLs (Fig 6.10). However, HPGE and ASA II show a slightly lower production as compared to RGE and ASA I. Reference lectins (Con A and PHA) do not show a remarkable NO production (Fig 6.10). Nitric oxide production by garlic lectins ranges from 1.5 to 2 μM as measured from the calibration curve. Nitric oxide induction by garlic lectins is not very significant. A similar trend has been observed even with potato extracts and potato lectin. Nitric oxide production is marginal and the result is not significant. Results of NO production by potato extracts and potato

lectin are presented in Fig 6.11. Potato extracts and potato lectin induce NO in the range of 0.5 to 1 μM.

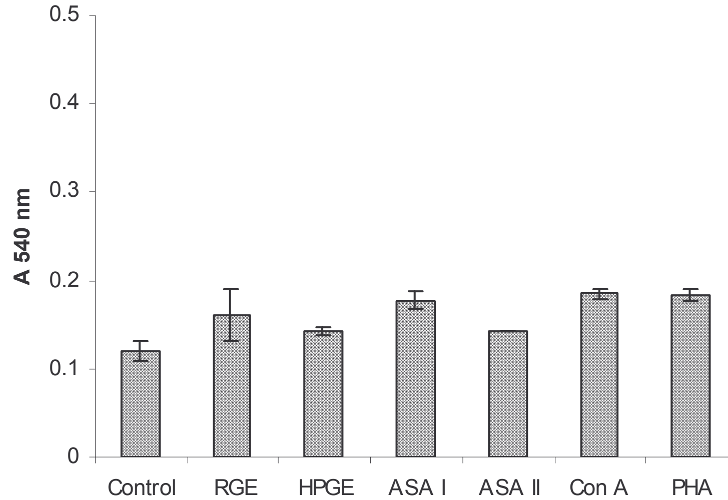


*Fig. 6.10. NO production from human PBLs by garlic extracts and lectins. Concentration of garlic extracts and lectins: 5 μg/mL. Absorbance of nitrate (proportional to released NO) is measured at 540 nm.*

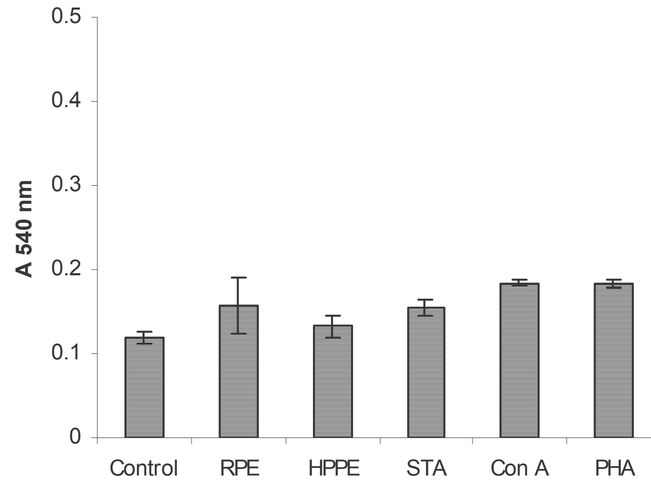


*Fig. 6.11. NO production from human PBLs by potato extracts and lectin. Concentration of potato extracts and potato lectin: 5 μg/mL. Absorbance of nitrate (proportional to released NO) is measured at 540 nm.*

### 6.4.8. Superoxide generation from rat PECs by garlic lectins and potato lectin

All the three purified lectins (ASA I, ASA II and STA) were checked for their ability to induce reactive oxygen species from isolated rat PECs containing

macrophages. The lectins were used in the concentration range of 0.1 to 10 μg/mL for stimulation. The results are shown in Fig. 6.12. All the three lectins do not show superoxide production, and only the raw garlic extract (RGE) at 10 μg/mL induces superoxide production (~3.5 fold compared to the control).

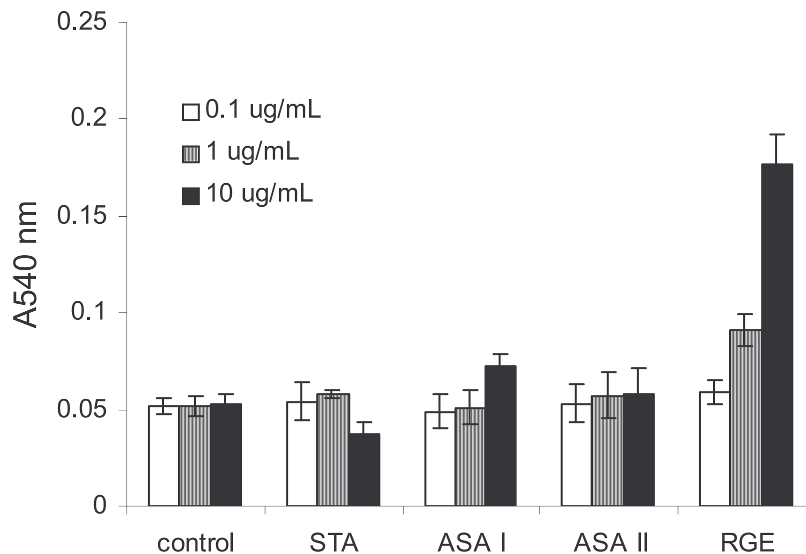


*Fig. 6.12. Superoxide generation from rat PECs in the presence purified garlic lectins and potato lectin. Concentration of garlic lectins, potato lectin and RGE: 1 to 10 μg/mL.*

## 6.5. DISCUSSION

Dietary lectins are plant protein components with the ability to bind, selectively, free or conjugated saccharide in a reversible way by two or more binding sites (Sharon, 1993). Some lectins have been shown to induce lymphocyte proliferation or modulate several immune functions, and these mitogenic lectins are useful as reagents to study the interactions of lectins with lymphocytes *in vitro* (Kilpatrick, 1999). They are able to induce cell division in different kinds of cells, and some plant lectins are mitogenic towards enterocytes. The *in vitro* mitogenicity of lectins is typically measured as their ability to induce proliferation of lymphocytes from lymph organs or blood; the best described and most used are PHA, PWM and Con A. An individual lectin can bind to

several glycoproteins on the lymphocyte surface, resulting in the interactions that may or may not be functionally relevant and that may have opposing effects. Studies with lectins and monoclonal antibodies have established that a surprisingly large variety of cell-surface molecules influence the initiation and regulation of lymphocyte activation and proliferation (Kilpatrick, 1999).

However, not all plant lectins are mitogenic; they can be grouped as mitogenic, non-mitogenic or antimitogenic. Actually, WGA has been found to be nonmitogenic (Muraille et al., 1999), antimitogenic (Barret et al., 1983), and mitogenic for either T cells or B cells (Kilpatrick, 1995), depending on the concentration of the lectin or the purity of the examined cells. The present chapter describes the interaction of two mannose specific lectins ASA I and ASA II from garlic bulbs, and a oligo-GlcNAc specific lectin STA from potato tubers on human and murine lymphocytes. An effort has been made to assess their mitogenic and co-mitogenic activities.

Garlic lectins ASA I and II induced proliferation of both human and murine lymphocyte population, and the degree of proliferation by ASA I is comparable to those of the reference lectins, Con A and PHA. ASA II shows about 40-50% less stimulatory effect on murine splenocytes and about 70% less in case of human PBLs. Among RGE and HPGE, only RGE shows a significant stimulatory effect against both human and murine lymphocytes. The varying stimulatory response between ASA I and ASA II might be because of their difference in the carbohydrate binding ability. ASA I is known to bind glycoproteins about 3-fold stronger compared to ASA II, as evidenced by glycoprotein binding assay and hemagglutination assay (details in Chapter 5). As stated earlier, glucose/mannose-specific lectins obtained from the seeds of the same tribe (Dioclea) had similar structures but were distinct in their ability to stimulate human lymphocytes (Barral-Neto et al., 1992). The subtle differences in the mitogenic activity of these lectins appeared to be due to a combination of diverse molecular forms that they present. Small differences could also be attributed to the amino acid sequence in the carbohydrate-binding site involved in sugar interaction that may affect the fine specificity for sugars in the cell membrane.

Mitogenic activities of plant lectins are consequences of their carbohydrate binding ability; the cell-surface glycoconjugates are involved in mitogenic process. Unlike typical antigens, which perhaps stimulate 0.01-0.1% of the lymphocyte population, mitogenic lectins can stimulate 20% or more (Kilpatrick, 1999). There have been indications that mitogenic lectins could be active both within the gut, and also systemically after entry by either alimentary or parenteral routes. It is clear that one of the most remarkable properties of lectins is their resistance to degradation within the alimentary canal; there is also evidence for this in human (Brady et al., 1978; Freed and Buckley, 1978: Kilpatrick et al., 1985) as well as in rodents. In rats, various dietary lectins act as powerful growth stimulators for the small intestine and after absorption can even affect remote organs like pancreas (Pusztai, 1991). Mitogenic lectins (con A, PHA, PWA) have little in common regarding saccharide specificity, but that would not exclude the possibility that different mitogenic lectins could bind to different saccharide structures on the same glycoprotein, so it is not obvious which interaction is functionally important.

Potato extracts as well as potato lectin do not show the mitogenic stimulation of human and murine lymphocytes. STA was found to be non-mitogenic at lower concentration, but at higher concentration shows inhibitory response for lymphocytes. The response shown by STA strongly agrees with earlier findings, where STA and tomato lectin have been found to inhibit lymphocyte proliferation induced by purified protein derivative (PPD) and other activators (McCurrach and Kilpatrick, 1988). Other lectins, such as wheat germ agglutinin (WGA) and datura lectin (DSA) have been shown to be anti-mitogenic (McCurrach and Kilpatrick, 1988). The solanaceae lectins and WGA have basically similar saccharide specificity, and it is likely that they all act as anti-mitogens by binding to the same receptor. This could be the physiological receptor for putative suppressor lymphokines (Greene et al., 1981) or for interleukin-2 (Reed et al., 1985).

Lectins are still the most widely and accessible reagents for activating lymphocytes in diagnostic work. The specificity of lectin-carbohydrate interactions has also encouraged active research due to efforts to design therapeutic analogues of

carbohydrates (Pani et al., 2000). The activation and mechanism of mitogenic stimulation by lectins still need to be understood with respect to the finer details. Though the initial step in mitogenic stimulation is binding of the lectin to the cell surface carbohydrate moieties, this alone is not sufficient, since certain lectins are non-mitogenic, even though they bind well to human lymphocytes (Ashraf and Khan, 2003). Thus, it is believed that mitogenic lectins interact with unique membrane components that may act as 'stimulatory receptors' and that non-mitogenic lectins may not bind to these membrane components, or alternatively bind to 'inhibitory receptors'.

Although some lectins are polyclonal activators both *in vivo* and *in vitro*, others may display a broad range of activities toward human lymphocytes. Indeed, the same lectin (eg. WGA and Datura lectin) may be mitogenic, co-mitogenic, or anti-mitogenic, depending on the experimental conditions (Kilpatrick, 1999). Several lectins (WGA, LEA, DSA, *A. bisporus* lectin) were surprisingly found to be anti-mitogenic (Greene and Waldmann, 1980; 1981); in other words they act to antagonize the stimulative activity of mitogens with which they are co-cultured (Nachbar et al., 1980; Kilpatrick et al., 1986). The non-mitogenic nature of these lectins could be explained by their blocking the natural function of CD45, a tyrosine phosphatase with an essential role in T-cell activation (Trowbridge and Thomas, 1994; Yakura, 1994; Klaus et al., 1996).

The 'mitogen receptor' is the T-cell receptor and associated molecules (T-cell receptor complex), and lectin activation is similar to antigen-induced activation (Chilson and Chilson, 1989). Lectins bring about a polyclonal activation *via* common saccharide structures. It would not be necessary for the lectin to bind the T-cell receptor directly, for the latter is associated on the lymphocyte surface with other glycosylated molecules, especially CD3, but also to some extent with CD2, CD4, CD5 and CD8 (Brown et al., 1989; Suzuki et al., 1992; Osman et al., 1992). With these considerations, it appears that mitogenic lectins/antigens bind to the T-cell receptor complex, whereas non-mitogenic lectins either do not bind to the complex or bind to other, accessory molecules essential to the transmission of signals. Mitogenic lectins cause signals leading to the

synthesis of interleukin 2 receptors (IL-2R); non-(anti) mitogenic lectins do not (Altman et al., 1990).

Garlic lectins and potato lectin were tested for their ability to induce nitric oxide production which is a signal molecule and serves as a secondary messenger for various biochemical signaling mechanisms (Nathan and Xie, 1994). All the three purified lectins did not induce NO production form human PBLs. However, it has been reported that fresh garlic powder increased cytochrome nitric oxide synthase (cNOS) activity; but equivalent quantity of arginine to that found in fresh garlic powder did not affect cNOS activity in experimental animals fed with garlic (Das et al., 1995; 1996). These lectins also do not possess the ability for the generation of reactive oxygen species, whereas RGE at higher protein concentration (10 μg/mL) induced ROS production by 3.5 fold. This implies that other components present in raw garlic have ROS stimulatory activity. .

## 6.6. SUMMARY AND CONCLUSSIONS

Both the garlic lectins were found to be mitogenic towards human peripheral blood lymphocytes (PBLs) and murine splenocytes as well as thymocytes. The proliferation effects shown by ASA I (70-75%) and ASA II (35-40%) are comparable to those of the reference mitogens Con A (100%) and phytohemagglutinin (PHA) (85%). On the other hand, potato lectin, raw- and heat- processed potato extracts did not show any significant proliferation effect on both human PBLs, and murine splenocytes & thymocytes (<10% compared to Con A). ASA I and ASA II were unable to induce reactive oxygen species (ROS) from macrophages; however, RGE shows a significant increase in ROS generation. Both potato and garlic lectins failed to induce the production of nitric oxide (NO) generation from human PBLs. From these observations, it is evident that garlic lectins possess mitogenicity for lymphocytes whereas potato lectin is devoid of the same. From the present study, it is evident that mannose-specific garlic lectins have stimulatory effect and potato lectin has inhibitory effect on lymphocyte proliferation. It can be concluded that garlic lectins are mitogenic and co-mitogenic, whereas potato lectin is non-mitogenic or anti-mitogenic in nature

# Chapter 7

# Assessment of immunogenicity and adjuvanticity of garlic lectins by their mucosal and systemic responses

## 7.1. INTRODUCTION

The trend towards an increased consumption of mainly processed food results in a higher intake of non-nutritive compounds such as lectins. The health effects of dietary lectins have been addressed by several investigators who have pointed out that currently only limited information is available on the immunomodulatory effects of lectins (Freed 1999; Kilpatrick, 1999; Cordain et al., 2000). Lectins are typical globular proteins that are mostly resistant to digestion in the gastrointestinal tract. They affect the integrity of the intestinal epithelium and the absorption of dietary antigens, and may further become internalized and circulate intact in the peripheral blood (Pusztai et al., 1993; Wang et al., 1998). Lectins provoke diverse biological consequences in mammals such as hyperplasia and hypertrophy of the small intestine and could increase the permeability of the intestinal wall, resulting in enhanced absorption for dietary co-administered antigens (Pusztai 1991; Cordain et al., 2000). Lectins are highly immunogenic and capable of inducing a specific immune response after oral administration, in contrast to other dietary proteins. Orally ingested lectins may, after absorption, affect the immune system systemically; however, it is also important to consider the local mucosally-induced immune response, as the mucosa is where orally ingested lectins initially encounter the immune system.

Mucosal and particularly oral administration of antigen is frequently ineffective at stimulating strong and sustained immune responses. In many cases, a number of high doses are required and the response induced may be of short duration (Mayer, 1998; Nossal, 1999). Delivery systems and adjuvants can enhance the responses elicited to mucosally administered antigen, for example, by protecting the antigen targeting to the epithelium. One strategy for antigen targeting is the use of molecules such as plant lectins, which bind specifically to mucosal epithelial cells. A number of plant lectins are found to be stable in the rodent gut and to interact with the mucosal epithelium after feeding (Pusztai and Bardocz, 1996). Of particular interest are studies that have shown selective labeling of antigen-sampling M cells in the mouse Payer's patch by fucose-specific lectins (Clark et al., 1993; Sharma et al., 1998). There has been evidence for

translocation of plant lectins across the gut in both mice and humans (Clark et al., 1995; Wang et al., 1998). The finding that certain plant lectins interact with the mucosal epithelium and are translocated across the gut may be exploited in vaccine delivery to induce mucosal and systemic immunity. Mitogenic plant lectins including phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM) are routinely used for activation of lymphocytes in vitro (Heegaard and Muller, 1988). High levels of specific serum IgG were induced by oral administration of tomato lectin in mice (Naisnett and Woodley, 1995). It has been suggested that, proteins with lectin or lectin-like properties are effective mucosal antigens, and a relationship between receptor binding in the gut and mucosal immunogenicity (Di Aizpurua and Russel-Jones, 1988) has been proposed.

The gut-associated lymphoid tissue (GALT) is highly selective with regard to recognition of antigens. The microenvironment prevents an immune response to food antigens by actively suppressing the function of immunocompetent cells. Perturbation of the microenvironment, e.g. by infection or toxic insults, may result in an immune response to orally-applied antigens, which in turn might contribute to the development of food allergy or chronic inflammatory disease in the gut (Watzl et al., 2001). In this context, the role of plant lectins, natural constituents of many cereals, legumes and vegetables, in the induction of secondary immune response is very important.

In recent years, medicinal applications have been found for many dietary proteins (Franssen et al., 1994). Some of these proteins have been used as vehicles for drug delivery, others for internal diagnostic processes and some for their intrinsic pharmaceutical properties (Hnatowich et al., 1987). Another important aspect is how they interact with the immune system. It has been shown that human serum contains natural antibodies to dietary proteins, which were never injected. It was also demonstrated that these antibodies can be very specific or polyreactive (Tchernychev et al., 1995). It is not known whether such natural antibodies have any physiological effects. These studies also demonstrated that, antigen stimulation of lymphoid tissue associated with the gut can generate potent local and systemic humoral immune

responses (Mestecky and McGhee, 1989). An aggressive environment of the digestive system can modify the structure of the ingested protein. Consequently, its antigenic properties change according to the intact, native antigen (Tchernychev et al., 2000).

Relatively few molecules have been identified that are able to induce a strong immune response when delivered by the oral or other mucosal route. Lectins are some of the few proteins that when given by the mucosal route induces an antibody response. Although still sparsely documented, the type of immune response (local vs. systemic, tolerance vs. immunity) may be strongly dependent on the site of absorption in the gut. Uptake across Peyer's patches (PP) might induce immunity, whereas, absorption through enterocytes might induce tolerance. Apart from indications that the binding activity of lectins can confer immunogenicity (Di Aizpurua and Rossel-Jones, 1988; Kjaer and Frokiaer, 2002), there are very few investigations on what determines mucosal immunogenicity of plant lectins.

However, mucosal delivery of non-replicating antigens generally does not stimulate strong immune response, requires multiple doses (Nossal, 1999) and may result in systemic unresponsiveness (McGhee and Kiyono, 1999). A number of strategies may be used to enhance responses to mucosally delivered vaccines including bacterial vectors (Ward et al., 1999), biodegradable microparticles, or liposomes (O'Hagan, 1998; Rogers and Anderson, 1998). The most powerful mucosal adjuvants identified to date are cholera toxin (CT), heat-labile enterotoxin (LT) and mistletoe lectin I (ML-1) (Elson and Ealding, 1984; Clements et al., 1988, Lavelle et al., 2001). Stimulation of toxin-specific local and systemic responses and responses to co-administered antigens after mucosal application distinguish these molecules from most soluble proteins (Williams et al., 1999). Although toxicity prevents clinical application, molecules with retained adjuvanticity and low toxicity have been generated.

Garlic is present in many foods as a flavor enhancer and is not a component in any vaccine or drug administered to humans. Garlic cloves contain a limited amount of protein, two of which (alliinase and mannose-specific lectins) together constitute more than 50% of the total amount of proteins. Therefore, in the present chapter an attempt

has been made to examine the two purified garlic lectins (ASA I and ASA II) to assess their ability to induce anti-lectin antibody response by both intradermal and intranasal routes of administration using BALB/c mice. The study will provide insight into the systemic and mucosal effect of immune responses for these lectins. The mucosal adjuvant effect of these two lectins was also assessed for a poor antigen ovalbumin (OVA) by co-administration of OVA with ASA I and ASA II. The present study will help to analyze the immunomodulatory effects of dietary garlic lectins with a focus on their immunogenicity potential and possible adjuvant activity.

## 7.2. MATERIALS

This study was undertaken after clearance by the Institutional Animal Ethics Committee (IAEC). The animals were maintained in the animal house facility of the department of Biochemistry and Nutrition, CFTRI, Mysore. All the necessary precautions and care were taken during the experiments as per the guidelines provided by the IAEC.

Garlic lectins used in the present study were purified in the laboratory (described in Chapter 5). Seven weeks old female BALB/c mice were procured from the central animal facility, Indian Institute of Science, Bangalore, India. Mice had access to a commercial stock diet (rat/mice pellet), and were housed as a group of six in a cage at the animal house facility. Ovalbumin (OVA; type V, hen egg), concanavalin A (Con A), and goat anti-mouse IgG-alkaline phosphatase conjugate were products of Sigma-Aldrich Co., St. Louis, MO, USA. Flat-bottom 96-well microtiter plates (MICROLON) were purchased from Greiner Bio-One GmbH, Frickenhausen, Germany. All other chemicals and reagents used in this study were of analytical grade

## 7.3. METHODS

### 7.3.1. Experimental animals

Young adult (8-12 weeks old) female BALB/c strain mice were used throughout these studies. Well-maintained hygienic quality food and water were provided to the animals. The diet used was a commercial special diet for rat and mouse maintenance,

the diet comprising primarily cereal products. The ambient temperature was maintained at 23 ± 3 °C and relative humidity was 55 ± 10% with a 12 h light/dark cycle. All experiments were carried out under the provisions of the Indian Animals Act.

**7.3.2. Preparation of antigens**

Garlic lectins ASA I and ASA II purified from garlic bulbs (as given in chapter 5) following extensive dialysis against water, were lyophilized. The dried lectin powder was dissolved in phosphate-buffered saline buffer (PBS), pH 7.4., and their concentrations were adjusted to 1 mg/mL based on Bradford protein assay. Commercially procured ovalbumin (OVA) was prepared in PBS at 1 mg/mL.

**7.3.3. Grouping of animals for immunization**

The animals were divided into four groups for both intradermal and intranasal immunization. The animals were randomly distributed considering the body weights. The average weights of the animals in each group were almost similar. The random distribution of animals was done to ensure the same response from animals in each group receiving the antigens. Each group contained six animals. The first group was a untreated control, to which only saline was administered to stress the animals. The second group received OVA, the third group received ASA I, and the fourth group received ASA II. All the antigens were administered by either intradermal or intranasal route at the same concentration following the standard protocol.

**7.3.4. Intradermal (systemic) immunization schedule**

BALB/c mice (n=6) received an intradermal injection of 30 μL of 1 mg/mL antigen on the dorsum of each ear on days 0 and 7; control animals were untreated (naïve) with any antigen and received only 30 μL of saline throughout the experiment (for stressing the animals as it was done for the treatment groups). Fourteen days following the initiation of the antigen exposure, all animals were exsanguinated by cardiac puncture. Blood was drawn from the animals of control and treated groups on day 7 and 14 by

retro-orbital vein puncture using heparinized capillary tube. Serum was separated and stored at -20°C.

### 7.3.5. Intranasal (mucosal) immunization schedule

Groups of mice (n=6) were mucosally immunized by intranasal (i.n.) route of administration. On days 1, 7, 14, 21, 28, 35 and 42 each group of mice was administered with one of the following: phosphate-buffered saline (PBS; for control group), OVA and garlic lectins (ASA I and ASA II), which were all prepared in PBS at a concentration of 1 mg/mL. For i.n. immunization, mice were initially anesthetized using mild dose of diethyl ether and were carefully monitored with an observation of heart beat, then samples were dosed through fine tips attached to a pipette, Thirty micrograms of OVA and plant lectins were delivered in 30 μL of PBS (15 μL was applied to each nostril during dosage administration). Fifty days following the initiation of the antigen exposure, all animals were exsanguinated by cardiac puncture. Blood was drawn from the animals of control and treated groups on day 13, 34 and 50 day by retro-orbital vein puncture using heparinized capillary tube. Serum was separated and stored at -20°C.

### 7.3.6. Body weights of experimental animals

The body weight of each individual animal in the experimental groups was monitored at specific intervals of time during the experiment. The weights were taken prior to the administration of each dose by intradermal or intranasal routes of administration. The change in animal weights reflects the sign of their growth and monitoring the body weights gives a measure of the effect of antigens on animal growth.

### 7.3.7. Collection of blood and serum separation

Blood was collected from the experimental groups by retro-orbital vein puncture using heparinized capillary tubes at specific intervals. On final day of the experiment ($14^{th}$ day for i.d. group and $50^{th}$ day for i.n. group), animals were terminally anesthetized

to collect blood. Mice were then sacrificed by anesthetic overdose followed by heart puncture. The collected blood was allowed to stand at room temperature for 1 h with loose cotton plugging for clotting. After the eppendorff tubes were transferred to 4°C for 1 h, they were centrifuged in the cold at 750 X g for 10 min. The clear yellowish serum was pipetted to a new eppendorff tube and stored at -20°C. This serum was used to check the anti-protein IgG antibody response against the intradermal/intranasal administration of antigens. .

**7.3.8. Calculation of spleen and thymus index**

The spleen and thymus of the individual animals of each group was collected on the final day after sacrificing the animals. Spleen and thymus were weighed both in control and treated animal groups. Based on the spleen and thymus weights and body weight of the individual animals, spleen index and thymus index were calculated using the following formulae: Spleen index = Spleen weight / Body weight, whereas for Thymus index = Thymus weight / Body weight; the index was expressed as mg/g.

**7.3.9. Detection of specific IgG antibodies by ELISA**

ELISA was performed for the detection of specific IgG antibodies. Sera at 1: 10 dilution were titrated in the appropriate dilution buffer. For the assay, microtiter plates were coated with 100 μL per well of 0.1 mg/mL antigens in 0.1 M carbonate-bicarbonate buffer, pH 9.6, and incubated at 4°C overnight. After washing with PBS containing 0.05 % Tween (PBS-T), plates were blocked with 2% gelatin/dilution buffer and incubated at 37 °C for 2 h. Plates were washed, and samples (mouse serum diluted 1:10 with a dilution buffer containing PBS-T with 1% BSA) were added and incubated at 4°C overnight. The serum samples derived from each individual animal or pooled for each experimental group were added to consecutive wells in duplicate. Following the incubation, plates were washed, and incubated with 100 μL/well of goat anti-mouse IgG conjugated to alkaline phosphatase at 1:1000 dilution as a source of secondary antibody. The secondary antibody diluent used was PBS-T containing 1% BSA and

0.5% gelatin. The plates were incubated at 37°C for 2 h. After extensive washing, plates received 100 μL of alkaline phosphatase substrate (p-nitrophenyl phosphate at 1 mg/mL prepared in 10 % diethanol amine buffer, pH 9.4). The plates were further incubated for 20 min and the reaction was arrested by the addition of 100 μL 3 N NaOH. The absorbance of the plates was read at 405 nm using microtiter plate reader.

### 7.3.10. Mucosal immunization schedule for adjuvant activity

Groups of mice (n=6) were immunized on days 1, 14, 21, 28, 35 and 42, with phosphate buffered saline (PBS), OVA (30 μg) alone, or OVA (30 μg) mixed with one of the following: ASA I (30 μg), ASA II (30 μg), Con A (30 μg), RGE (30 μg). While restrained, mice were dosed with 30 μL of each preparation (15 μL placed over each nostril) using fine tips attached to pipette. Mice were held in place until the liquid was inhaled and were slightly anesthetized with low dose of diethyl ether. Blood samples were collected 1 day before immunization on days 14 and 35. Animals were terminally anesthetized to allow blood collection. Mice were then sacrificed by anesthetic overdose followed by exsanguination. Collected blood was processed to obtain the serum, which was stored at -20°.

### 7.3.11. Statistical analysis

Data are expressed as the arithmetic mean and standard deviation. Unpaired two-tailed t-test was used to test for significance between the groups. A paired t-test for means was used to test for significance in the same group at different time points.

## 7.4. RESULTS

### 7.4.1. Body weights of animals in the systemic and mucosal immune response

The body weights of the animals in the control and treated groups were carefully monitored. Animals were weighed prior to each dose administration. The average weight of the animals in the groups fell in the range of 25 ± 1 g. Weights of animals in the groups administered by intradermal route are summarized in Table 7.1.

**Table 7.1. Body weights of experimental animals at various times in the intradermal administration experiment**

| Samples* administered | Body weight (in g)* | | |
|---|---|---|---|
| | Day 0# Mean ± SD | Day 7 Mean ± SD | Day 14# Mean ± SD |
| Control | 24.46 ± 1.51 | 24.70 ± 1.57 | 25.00 ± 2.32 |
| OVA | 25.26 ± 2.98 | 25.46 ± 2.91 | 25.62 ± 3.28 |
| ASA I | 25.82 ± 2.20 | 25.47 ± 1.95 | 25.50 ± 1.40 |
| ASA II | 25.70 ± 1.24 | 26.03 ± 1.34 | 25.65 ± 1.56 |

* n=6 in each group; the body weight is expressed as mean weight of animals in a group
# not significant at $p < 0.05$

By intradermal route of administering the garlic lectins or OVA, there is no significant change in the body weights of animals in the treated group as compared to control group. Though there is a slight increase in the body weight of ~ 0.5 to 1 g in the control group, and only a slight change in the treated group, the results are not very significant. This clearly shows that the dietary garlic lectins and OVA do not have any effect on the growth and body weight of animals when administered by intradermal route.

Weights of animals in the groups administered by intranasal route are shown in Table 7.2. By intranasal route of administration of garlic lectins and OVA, only control group shows a significant ($P < 0.05$) increase in weight of 1-2 g. The treated group does not show any significant increase or decrease in body weights. The slight changes in the weights of treated group from day 0 to day 50 may be due to difference in the metabolic activity of animals in different treated groups. These observations clearly indicates that the body weights of animals remain fairly constant after the administration

of garlic lectins by intranasal route suggesting that the garlic lectins are not growth-retardant, and are not toxic.

**Table 7.2. Body weights of experimental animals at various times in the intranasal administration experiment**

| Samples administered* | Body weights (in g)* | | | |
|---|---|---|---|---|
| | Day 0# Mean ± SD | Day 14 Mean ± SD | Day 35 Mean ± SD | Day 50# Mean ± SD |
| Control | 25.33 ± 3.29 | 25.73 ± 4.50 | 26.17 ± 3.57 | 27.03 ± 2.74 |
| OVA | 25.58 ± 1.55 | 26.24 ± 2.41 | 26.94 ± 2.13 | 26.80 ± 2.56 |
| ASA I | 25.68 ± 1.39 | 25.24 ± 1.86 | 25.28 ± 1.36 | 25.22 ± 1.59 |
| ASA II | 25.44 ± 1.26 | 25.66 ± 0.85 | 25.56 ± 0.89 | 25.38 ± 1.33 |

* n=6 in each group; the body weight is expressed as mean weight of animals in a group
#not significant at $p < 0.01$

**7.4.2. Systemic anti-lectin immune response**

The effect of OVA and garlic lectins (ASA I and ASA II) was studied without adjuvant on the systemic immune response, by intradermal administration in BALB/c mice. OVA is a well-known poor antigen and hence serve as a control protein. The anti-lectin IgG response in comparison to the response from OVA provides a measure of the systemic response of garlic lectins. The anti-lectin IgG response by intradermal route of administration is shown in Fig. 7.1. The IgG response to OVA on days 7 and 14 are similar, and there is no significant increase in anti-OVA IgG on day 14 as compared to day 7. However, both lectins ASA I and ASA II showed a significant increase in anti-lectin antibodies on day 14 compared to day 7. The anti-lectin antibody response on day 14 was found to be higher (significant at the level of $P < 0.005$) for both ASA I and ASA II as compared to control protein OVA indicating that garlic lectins are strong systemic immunogens. IgG response to ASA I on day 14 is very marked as it shows

~3.5 - 4 fold increase in comparison to OVA response (Fig. 7.2). However, ASA II shows only a 2.5 fold higher IgG response compared to the response shown by OVA.

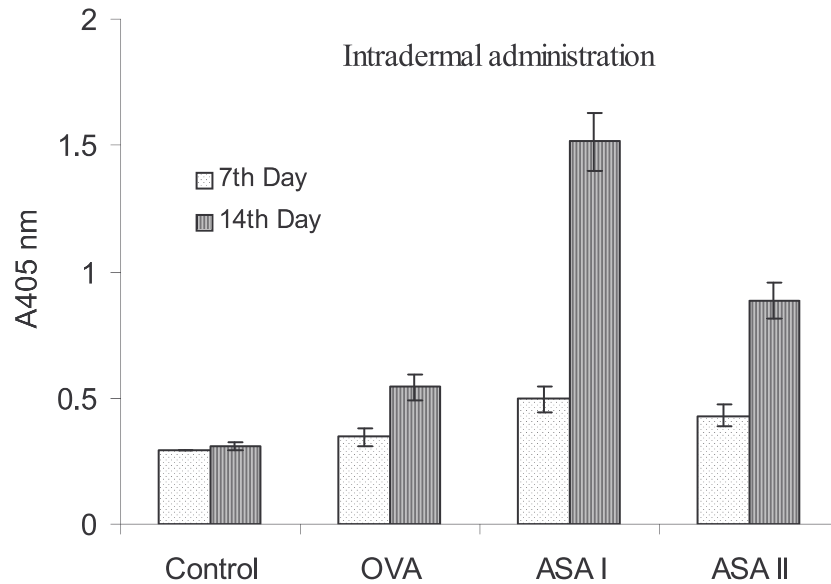


***Fig. 7.1.*** *Systemic immune (IgG) response against proteins (OVA, ASA I and ASA II) measured from the serum of animals (BALB/c mice) administered with OVA, ASA I, or ASA II by intradermal route. Amount of antigen: 10 μg/well. Serum dilution, 1:10 (volume: 100 μL/mL).*

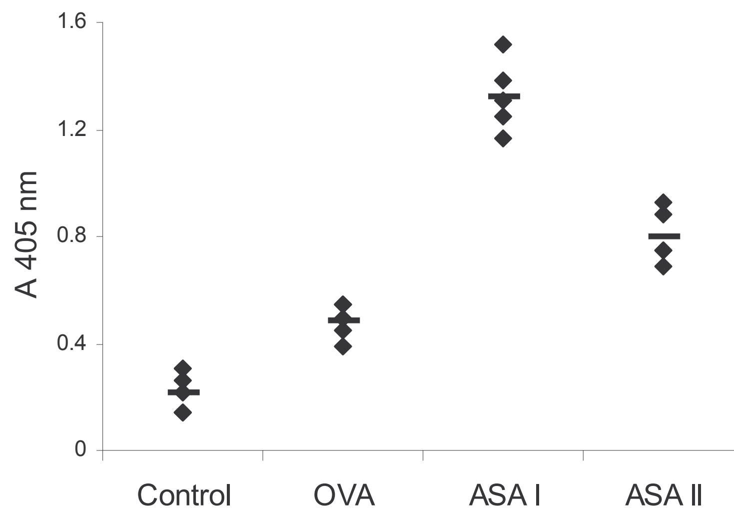


***Fig. 7.2.*** *Anti-protein IgG response on day 14$^{th}$ of intradermal administration of antigens to BALB/c mice. Antigen amount: 10 μg/well. Serum dilution, 1:10 (volume:100 μL/mL).*

### 7.4.3. Mucosal anti-lectin immune response

OVA and garlic lectins were administered by intranasal route to study the mucosal immune response. Serum obtained at different time periods was estimated for anti-protein IgG response and the results are shown in Fig. 7.3. The anti-lectin IgG response on day 14 is represented in Fig. 7.4.

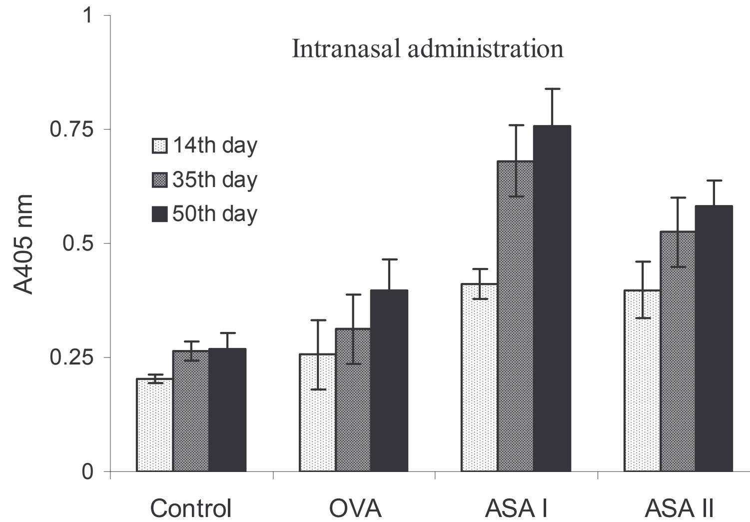


***Fig. 7.3.*** *Anti-protein IgG response against proteins (OVA, ASA I and ASA II) measured in the serum of animals (BALB/c mice) administered with OVA, ASA I and ASA II by intranasal route. Antigen amount: 10 μg/well. Serum dilution, 1:10 (volume 100 μL/mL).*

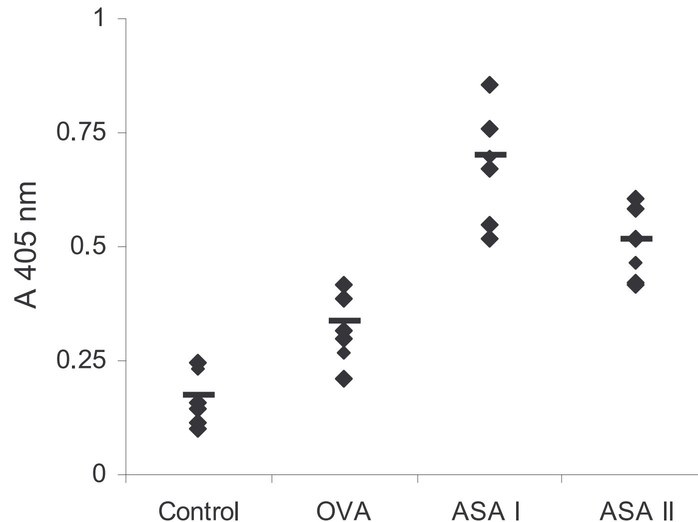


***Fig. 7.4****. The anti-protein IgG response on day 50 of intranasal administration of antigens to BALB/c mice. Amount of antigen: 10 μg/well. Serum dilution, 1:10 (volume 100 μL/mL).*

OVA shows a marginal increase in anti-OVA IgG response compared to naïve control group and there is a slight increase in the response from day 14 to day 50, which indicates that the anti-OVA response increase in relation to dose of administration; the increase in response to dose is significant at $p < 0.01$ and is not significant at the level of $p < 0.001$. The anti-lectin IgG response to garlic lectins is significantly higher as compared to the response for OVA (significant at $p < 0.001$). The mucosal IgG response to ASA I is markedly strong (2-2.5 fold increase), whereas in the case of ASA II, the response is 1.75 to 2-fold higher as compared to OVA group. These results indicate that ASA I is a stronger mucosal immunogen compared to ASA II. This may be due to the difference in their binding ability to cell surface glycoproteins involved in immune response.

### 7.4.4. Spleen and thymus weights and their indices in control and treated groups

The spleen and thymus were isolated on day 14 and 50 after sacrificing the animals in the control and treated groups. Their weights were measured to assess the activation of spleen or thymus by the treated antigens (OVA, ASA I or ASA II). The results are summarized in Table 7.3.

Table 7.3. Spleen and thymus weights (in mg) after administration of test samples by intradermal and intranasal routes

| Samples | Intradermal route* | | Intranasal route# | |
|---|---|---|---|---|
| | Thymus (in mg) Mean ± SD | Spleen (in mg) Mean ± SD | Thymus (in mg) Mean ± SD | Spleen (in mg) Mean ± SD |
| Control | 43.66 ± 1.25 | 105.33 ± 08.37 | 39.20 ± 5.58 | 106.03 ± 10.53 |
| OVA | 47.33 ± 7.03 | 133. 83 ± 07.77 | 41.70 ± 2.65 | 119.28 ± 08.91 |
| ASA I | 54.66 ± 2.49 | 136. 16 ± 05.01 | 49.37 ± 4.85 | 127.28 ± 25.66 |
| ASA II | 52.33 ± 3.29 | 129.16 ± 11.60 | 43.53 ± 1.64 | 118.15 ± 19.10 |

*spleen and thymus obtained on day 14
#spleen and thymus obtained on day 50

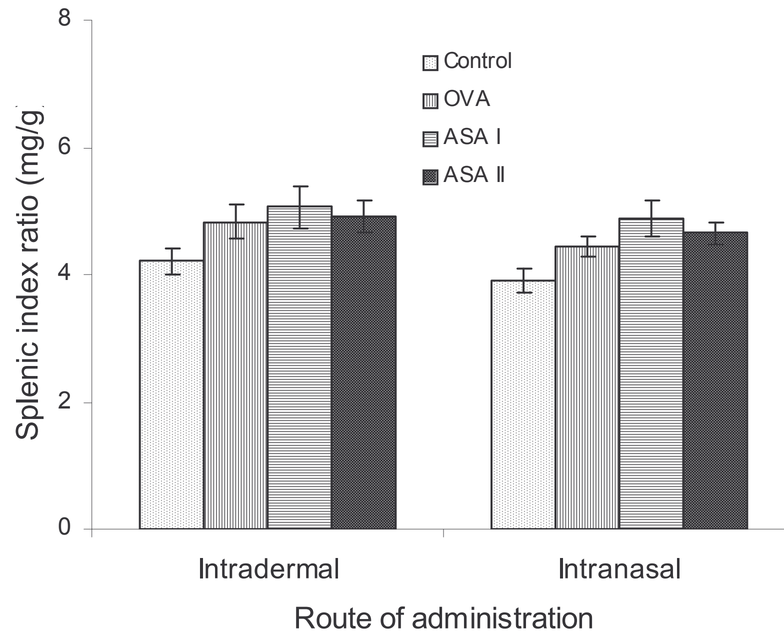


***Fig. 7.5****. Splenic index values in BALB/c mice administered OVA, ASA I or ASA II by intradermal or intranasal routes*

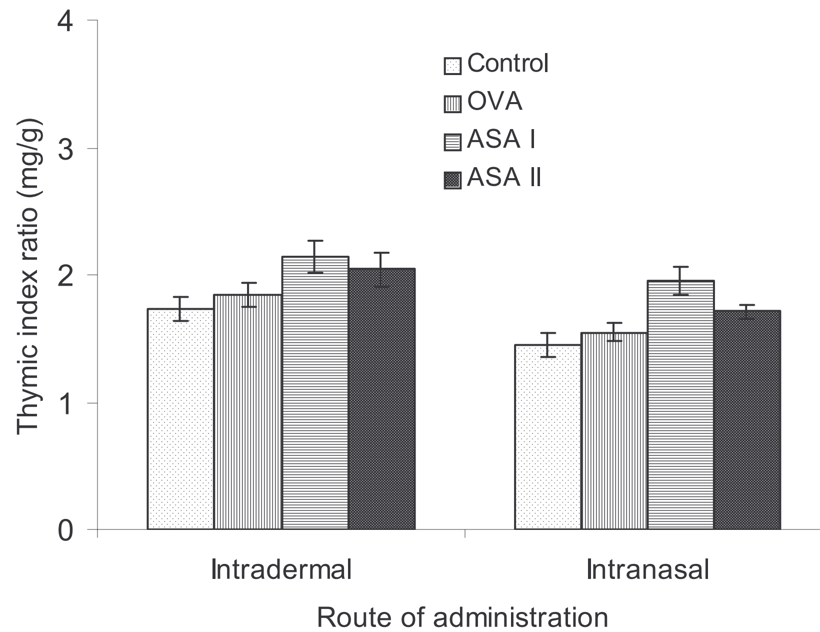


***Fig. 7.6.*** *Thymic index values in BALB/c mice administered OVA, ASA I or ASA II by intradermal or intranasal routes*

The results indicate that there is a considerable difference in the thymus and spleen weights in the treated group as compared to the untreated control group. The differences in weight of treated groups are significant only at $p \leq 0.01$. The splenic and thymic index calculated based on the spleen and thymus weights in relation to their body weights in each group indicates that there is no significant increase in the splenic and thymic index ratio between the treated groups. Though a slight increase in the indices was observed in case of ASA I-treated group by both intradermal and intranasal routes of administration, the results are not significant at $p \leq 0.001$ as compared to OVA group.

### 7.4.5. Mucosal adjuvant activity of garlic agglutinins ASA I and ASA II

Garlic lectins ASA I and ASA II were examined for their ability to induce both systemic and mucosal immune responses by intradermal and intranasal routes of administration. Both the lectins were found to be highly immunogenic as compared to protein OVA. These lectins were further analyzed for their ability to enhance IgG antibody response against a co-administered poor antigen OVA to assess their adjuvanticity.

#### 7.4.5.1. Body weights of animals in the adjuvanticity study

The individual animal in control and treated groups were compared for any change in body weights after intranasal administration of OVA alone or OVA with garlic antigens during the study of mucosal adjuvant effect. The results are shown in Table 7.4. There are no significant changes in the body weight of animals in the treated group as compared to the untreated control group. A slight decrease in the body weight of the group treated with OVA+ASA I, and OVA+Con A is observed but the decrease is not significant at $p \leq 0.005$. The observations indicate that garlic components administered by intranasal route for the treated groups are not toxic, and do not have any effect on the growth of the animals. This indicates that ASA I and ASA II are not growth-retardant proteins.

**Table 7.4. Body weights of animals at different time periods following intranasal administration of antigens in the adjuvanticity study**

| Samples administered* | Intranasal route Body weights (in g) | | | |
|---|---|---|---|---|
| | Day 0# Mean ± SD | Day 14 Mean ± SD | Day 35 Mean ± SD | Day 50# Mean ± SD |
| Control | 24.08 ± 1.78 | 26.03 ± 1.36 | 25.60 ± 1.59 | 25.83 ± 1.91 |
| OVA | 24.26 ± 1.56 | 23.75 ± 2.05 | 24.25 ± 2.28 | 24.70 ± 2.93 |
| OVA + ASA I | 24.45 ± 1.99 | 24.05 ± 1.65 | 24.30 ± 2.47 | 23.90 ± 2.90 |
| OVA + ASA II | 23.86 ± 2.11 | 24.46 ± 1.45 | 25.05 ± 1.76 | 25.75 ± 1.81 |
| OVA + Con A | 24.51 ± 1.30 | 23.90 ± 2.51 | 24.35 ± 3.02 | 24.30 ± 1.96 |
| OVA + RGE | 24.58 ± 1.51 | 24.53 ± 1.10 | 25.81 ± 1.53 | 25.95 ± 1.34 |

*intranasal administration of each antigen at 30 μg/30 μL concentration
#not significant at $p < 0.05$.

#### 7.4.5.2. Anti-OVA IgG antibodies in the adjuvanticity study

To study the mucosal adjuvant effect of purified garlic lectins ASA I and ASA II, OVA and OVA along with garlic proteins were administered to BALB/c mice by intranasal route. OVA is used as an experimental antigen and the IgG response against OVA was measured to check the adjuvant activity of garlic lectins. OVA was co-administered with garlic lectin ASA I or ASA II. Anti-OVA IgG response at different time periods of the treatment (day 14, 35 and 50) is shown in Fig. 7.8.

Anti-OVA IgG response in the sera of control and the test groups at day 50 after sacrificing the animals is shown in Fig. 7.8. Con A, the prototype lectin having glc/man specificity, and RGE containing all the components of whole garlic were also used for comparing the *in vivo* effects of garlic lectins by mucosal route.

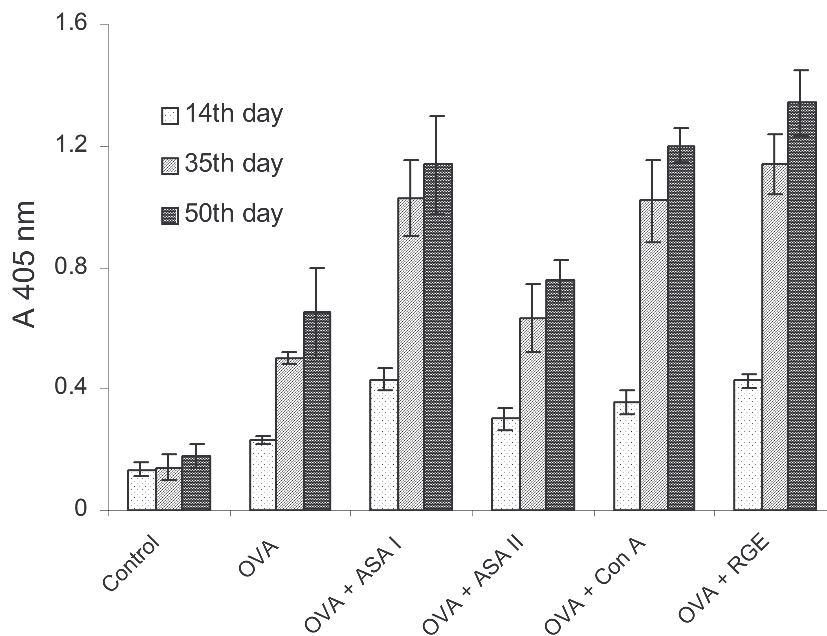


***Fig. 7.7**. Anti-OVA IgG response against OVA alone, and OVA with garlic components or con A at different times of dose administration. Coating antigen: 10 $\mu$g (100 $\mu$L of 0.1 mg/mL concentration); serum dilution, 1:10.*

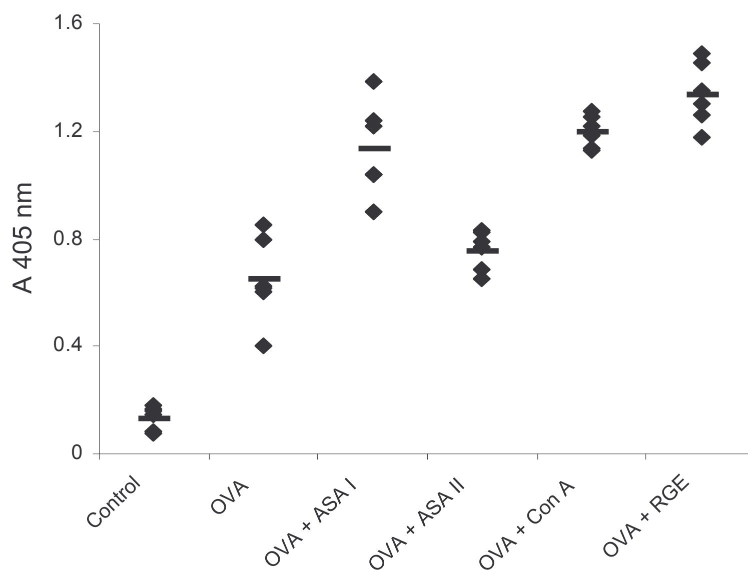


***Fig. 7.8.** Anti-OVA IgG response in the control and treated groups by intranasal route of administration. OVA was used as a model co-administered antigen for garlic lectins. The response shown is from the sera (at 1:10 dilution) collected on day 50. Antigen (OVA) amount coated: 10 $\mu$g.*

In the test group where OVA was administered with garlic lectins in the dose, ASA I group shows enhanced anti-OVA IgG response as compared to the response in the group where OVA alone was administered. The IgG response seen in OVA+ASA I group was significantly higher as compared to OVA+ASA II group. The OVA+ASA I group shows ~2 fold higher (significant at $p \leq 0.005$) anti-OVA IgG response compared to OVA group (Fig. 7.8). However, OVA+ASA II group shows only a marginal increase which is not significant at $p \leq 0.005$ as compared to OVA group. The OVA+con A group shows similar results as that of OVA+ASA I group. The OVA+RGE group shows maximum anti-OVA response, and is slightly more compared to ASA I and con A groups (Figs. 7.7 and 7.8).

### 7.4.5.3. Anti-OVA antibody titre in control and treated groups

The OVA-specific IgG response in control and treated groups was measured for its titre by serial dilution of serum. The results are shown in Fig. 7.9.

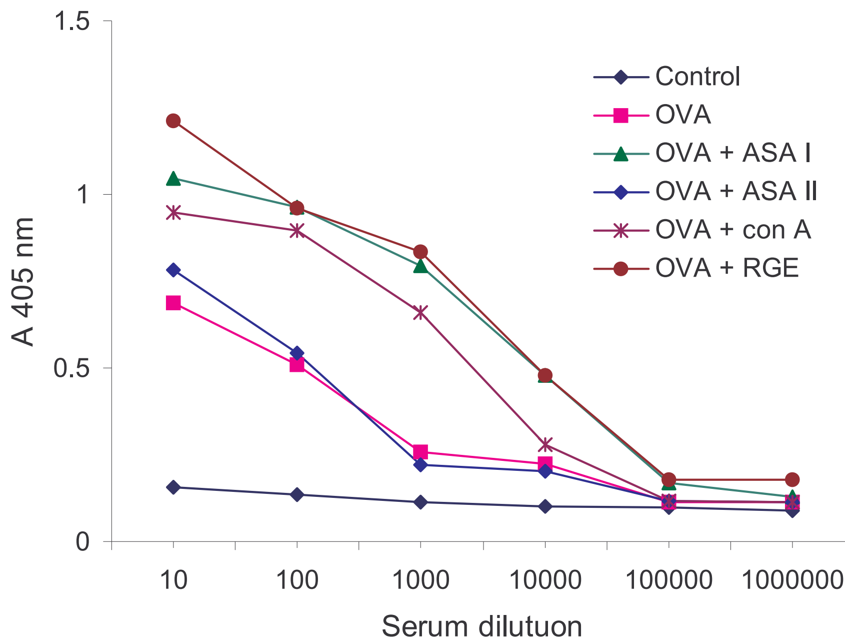


***Fig. 7.9.*** *The anti-OVA antibody titre (ELISA value) for control and treated groups. The initial dilution started with 1:10 dilution, and was subsequently serially diluted by 10-fold.*

The antibody titre from the sera collected on day 50 after sacrificing the animals of both control and treated groups were measured. It was observed that the anti-OVA IgG response was stronger in OVA+ASA I and OVA+con A groups as compared to OVA and OVA+ASA II groups. Detectable antibody titre was observed at 1:10000 serum dilution only for OVA+ASA I, OVA+con A, and OVA+RGE groups. For OVA and OVA+ASA II, groups, significant antibody detection was seen only at 1: 100 serum dilution. These observations clearly indicate that ASA I, Con A and RGE enhance the antibody production against co-administered antigens and can act as an useful adjuvant to boost the immune response against the weak immunogenic antigen, namely, OVA.

## 7.5. DISCUSSION

The gastrointestinal tract being the largest immunologic organ in the body possesses the greatest surface area exposed to the external environment, and is confronted with the largest antigenic load in the form of dietary proteins, commensal organisms and pathogens (Mayer, 2003). The mucosal immune system of the gut has the extraordinary ability to distinguish between the foreign toxic proteins and safe nutrient proteins (Chehade and Mayer, 2005). The GALT is highly selective with regard to recognition of antigens. The microenvironment prevents an immune response to food antigens by actively suppressing the function of immunocompetent cells.

The data available from the current literature demonstrate that intradermal or intranasal administration of immunogenic proteins induces vigorous IgG antibody responses. The present study shows that certain plant lectins such as garlic lectins ASA I and ASA II are mucosal immunogens, stimulating systemic and mucosal antibody responses after intradermal or intranasal administration, respectively. While delivery of the two lectins (ASA I and ASA II) stimulates higher antibody response than OVA, the magnitude of the response was different. After immunization with the garlic lectins and OVA, the body weights of the animals were monitored. The lectins did not affect the growth of animals and showed no adverse or toxic effects on animals indicating that the

intradermal or intranasal delivery of garlic lectins does not have any deleterious effects on the animals, but produces comparable immune responses.

OVA is a well-known antigen with weak immunogenic property (Oshiba et al., 1996). Garlic lectins were compared with OVA for their systemic IgG response upon systemic or mucosal delivery. Observations from this study indicates that anti-ASA I response is ~2.5 to 3.5 fold higher as compared to reference antigen (OVA) response by both systemic and mucosal routes of administration suggesting that ASA I is a strong immunogen. Anti-ASA II (~1.25 to 2 antibody response) is almost comparable to and only slightly higher than OVA response, suggesting that ASA II is a weak immunogen. Since ASA II shows about three fold lower binding ability to glycans (Chapter 5, § 5.4.5), it appears that the binding ability confers immunogenicity. Currently, there is little information on what determines the immunogenicity of lectins (Lavelle et al., 2000). For mucosal immunogenicity, it appears that lectins exert number of immunomodulating effects including the enhancement of antigen presentation by their effect on co-stimulation, promoting B-cell isotype switch determination, and enhancing T-cell priming efficiency against unrelated antigens. It has been suggested that lectins taken up across villous enterocytes enters the circulation to induce IgG responses in the spleen, while uptake across the Peyer's patch-follicle associated epithelium induces a local IgG and IgA response in the Peyer's patches and intestinal lymphoid follicles. The receptors to which lectins bind in the gut are poorly understood, but may be key factors in determining the mucosal immunogenicity.

Potato lectin has been found to be immunogenic in mice (Dearman et al., 2003), inducing IgG response by intraperitoneal (i.p.) administration. Potato lectin appears to be lacking a significant sensitization potential in humans based on its failure to stimulate IgE antibody response following i.p. treatment. Unlike peanut lectin, potato lectin did not induce significant IgE production, and this demonstrates that all lectins may not stimulate IgE production (Dearman et al., 2003). In addition, potato lectin has been demonstrated to be very stable to digestion in simulated gastric fluid (SGF) (Atherton et al., 2002; Dearman and Kimber, 2001). In another study, mucosal immunogenicity of five lectins

was studied by oral and i.n. delivery (Lavelle et al., 2000). Mistletoe lectin (ML-1), LEA, PHA, WGA and *Ulex europaeus* (UAE-1) lectin stimulated the production of specific serum IgG response. The study by Lavelle et al. (2000) concludes that highly immunogenic plant lectins when administered by oral or i.n. routes can effectively stimulate systemic and mucosal antibody responses.

Some studies have indicated that dietary lectins are stable in the digestive tract and binds to rat intestinal villi (Kilpatrick et al., 1985; Naisbett and Woodley, 1995). Data from another study confirms that some dietary lectins are immunogenic by oral route and demonstrated that they also stimulate systemic and mucosal responses when delivered by intranasal route (Lavelle et al., 2000). Higher immune responses were measured in the intranasally immunized mice, particularly after a single antigen dose, possibly due to strong binding of the delivered lectin to mucous lining in the gut. Mouse intestinal and Peyer's patch M cells are distinguished by expression of particular oligosaccharide moieties, which facilitate the interaction of lectins, and in turn, the process facilitates the internalization to bring out systemic immune response (Roth-Walter et al., 2004).

The present study exploring the mucosal adjuvant property of garlic lectins ASA I and ASA II, co-administered with OVA by intranasal route, showed an increase in anti-OVA specific IgG response for the OVA+ASAI group as compared to OVA alone and OVA+ASA II groups. The results indicate that only ASA I possesses mucosal adjuvant activity. Raw garlic extract along with OVA shows somewhat high IgG response compared to the purified garlic lectins; this may be due to the adjuvant effect of some other garlic components in addition to the effect of lectins in the extract. If adjuvanticity is linked to receptor binding, the effects in different species may vary due to differences in glycoconjugate expression. The factors that dictate mucosal immunogenicity are not fully understood although the ability to bind to cells is regarded as important (Nashar et al., 1996). However, a number of proteins with affinity for molecules on the surface of eukaryotic cells are not strong oral immunogens in mice (Elson and Ealding, 1984). In contrast to the present findings, intranasal administration of PHA+OVA has been demonstrated to elicit a lectin specific antibody response, but did not change the OVA-

specific mucosal or systemic antibody response (Lavelle et al., 2001). Different routes of administration may have an impact on the outcome of an immune response, since there are differences in the nasal and gut lymphoid tissues. This indicates that lectin or lectin-like properties do not necessarily confer potent mucosal immunogenicity. DNP conjugates of a number of plant lectins with different sugar specificities were of comparable efficacy after oral delivery in inducing haptenic mucosal immunogenicity (Di Aizpurua and Rossell-Jones, 1988; Heegaard and Muller, 1988), so lectin-antigen conjugates (covalently-coupled lectin-antigen molecules) may effectively stimulate immune response against the antigen even if the carrier lectin used is not highly immunogenic.

Dietary lectins can enter into systemic circulation and stimulate or inhibit the activation of immune cells depending on their lectin-cellular interactions. Dietary lectins can also make a passage for the co-existant poorly antigenic dietary proteins, and can act as adjuvants in boosting the specific immune response against co-administered antigens. Garlic lectins, in particular ASA I, exhibit strong systemic responses by both intradermal and intranasal administration. Results presented in the previous chapter (chapter 6) clearly show that ASA I produces a marked stimulation of splenocytes and thymocytes. Hence, it can be logically stated that ASA I is a strong dietary immunogen from garlic and also possesses mucosal adjuvant effect on co-administered food antigens. From the present observations, it can be clearly inferred that garlic lectins, upon mucosal administration, can trigger both systemic and mucosal immune responses.

In conclusion, it appears that garlic lectins, in particular ASA I, have the potential for use as adjuvants in boosting the immune response of co-administered antigens such as vaccines since garlic lectins are derived from a dietary source and are not toxic. To enhance the efficacy of new generation of (generally) poorly immunogenic recombinant ‘subunit’ vaccines, there is an increase demand for adjuvants and delivery systems that are effective when administered by mucosal routes. To date, the most effective mucosal adjuvants are the lectin subunits of A-B toxins from *Vibrio cholerae* (cholera toxin; CT)

and *Escherichia coli* (heat-labile enterotoxin; LT) and their derivatives. Some inorganic compounds like alum (gel containing derivatives of aluminum salts), aluminum phosphate and aluminum hydroxide have also been in use as an adjuvant for IgG antibody response against poorly immunogenic antigens (Wheeler and Woronoecki, 2001; Brewer, 2006).

### 7.6. SUMMARY AND CONCLUSIONS

By intradermal administration, ASA I shows 4.2 fold increase, and ASA II shows 2.8 fold increase in IgG response as compared to the control protein ovalbumin (a poor antigen). Both the lectins were found to be potent immunogens that indicates the systemic measure of immune response. By intranasal route, only ASA I (3.5 fold) shows a significant increase in IgG response than ASA II (1.2 fold) compared to control protein ovalbumin, which indicates the mucosal immune response. The anti-lectin IgG response for ASA I and ASA II is significant as compared to the control protein ovalbumin. During the administration of lectins, there was no reduction in the body weight of animals, suggesting that garlic lectins are not growth-retardant. These observations indicate that dietary garlic lectins are potent immunogens. The mucosal adjuvant potential of ASA I and II was assessed using the poor antigen OVA; only ASA I was found to have significant adjuvant activity whereas ASA II does not. It appears that garlic lectins are potent immunogens, in particular ASA I, have the potential for use as adjuvants in boosting the immune response of co-administered antigens.

# General summary and conclusions

Several dietary components are known to affect various functions of the immune system and to interfere with immune regulatory circuits. The immunological activity of carbohydrate-binding proteins of plant origin, the lectins, has long been recognized. Some plant lectins have been shown to be able to modulate important immune functions, such as inflammatory reactions and effector functions. Lectins are proteins or glycoproteins of nonimmune origin, which bind specifically to the glycan part of glycoconjugates in a sugar-specific manner. By binding to specific carbohydrates on cell surfaces, lectins can elicit multiple changes in cell and body metabolism.

Human food contains lectins, and lectins are therefore consumed in their native form when foods are eaten raw or when foods containing lectins that are heat stable are eaten. Because most lectins are resistant to digestion, they reach the small intestine in an active form. Lectins have many biological activities as they bind to carbohydrate on various cells. Many receptors are membrane integrated glycoproteins and lectin binding to these receptors evokes a variety of systemic and local effects such as cell division and growth, cell maturation, and cell death. The use of lectins in immunology as polyclonal activators has long been recognized as their binding to certain immune cells induces mitogenesis. Dietary lectins may be immunomodulatory and affect both the innate and adaptive immune response. Some lectins are able to induce a lectin-specific immune response, while others function as adjuvants, giving rise to an immune response against co-administered proteins. Inflammatory cytokine production, oral tolerance, production of antibodies and apoptosis are some of the immune functions influenced by dietary lectins.

The study presented in the thesis is focused on the modulatory effects of certain purified dietary lectins on the cells of the immune system (basophils, mast cells and lymphocytes), which will aid in delineating the role of lectins in non-allergic food hypersensitivity reactions (false food allergy), and their modulatory effects on certain immune cells to induce immunogenicity. Since lectins are often the major proteins in many plant foods and are found to interact with the surface glycans of many organ systems, it is appeared interesting to examine the modulatory aspects of some dietary

lectins to understand their importance and role in relation to hypersensitivity and immunogenicity.

Horse gram (*Dolichos biflorus*) is widely consumed in the tropical south Asian countries including rural areas of India. Since *Dolichos biflorus* agglutinin (DBA) is an important dietary lectin in horse gram, a study has been made of its effect on the degranulation of mast cells and basophils of atopic subjects. Among the atopic group, 10 of 48 subjects (21%) were found to be positive by SPT to DBA, and none were positive in the non-atopic group (n=20). Two subjects out of the ten who tested positive for DBA by SPT were found to be sensitized to DBA as revealed by the presence of specific IgE by ELISA and dot-blot. Histamine release was found to be 2-3 fold higher in DBA-allergic subjects than in non-atopic and atopic subjects. Basophil HR by DBA was found to be similar in both non-atopic and atopic subjects. However, DBA induces activation of mast cells *in vivo* in a sub-population (21%) of atopic subjects (translates to 4-5 % of general population). Two subjects have been identified as having food allergy to horse gram based on the presence of DBA-specific IgE with a positive correlation to basophil HR. This is the first study of food allergy to horse gram, and DBA has been identified as an allergen, and designated Dol b Lectin.

Potato lectin (*Solanum tuberosum* agglutinin, STA) is an unusual glycoprotein containing approximately 50% carbohydrates by weight. Of the total carbohydrates, 92% is contributed by L-arabinose, which are *O*-linked to hydroxyproline residues. The ferric chloride-orcinol assay (Bial's test), which is specific for pentoses, has not been used for the detection of pentoses in bound form as it occurs in Solanaceae lectins (potato, tomato, and datura lectins). Utilizing the pentose colorimetric assay for monitoring the presence of potato lectin, a simpler and shorter procedure for the purification of this lectin from potato tubers has been developed. The hemagglutination inhibition assay using glycoproteins revealed its binding to glycoproteins. Binding of potato lectin to chitobiose core of N-linked glycoproteins has been confirmed by glycoprotein-binding assay using horseradish peroxidase and avidin-alkaline phosphatase in ELLSA (enzyme-linked ligand sorbet assay) format. Heat-processed

potato extract (HPPE) was observed to retain ~50 % of the biological activity as compared to raw potato extract. The effect of potato lectin (STA) for its ability to release histamine from basophils *in vitro* and mast cells *in vivo* from non-atopic and atopic subjects has been studied. SPT on 110 atopic subjects using STA showed 39 subjects positive (35%, translates to 7-9 % general population); however, none showed STA-specific IgE and among 20 non-atopic subjects, none were positive by SPT. Maximal histamine release was found to be 65% in atopic subjects (n=7) as compared to 28% in non-atopic subjects (n=5); the release was specifically inhibited by oligomers of *N*-acetyl-D-glucosamine and the release correlates well with serum total IgE levels ($R^2$ = 0.923). Since potato lectin activates and degranulates both mast cells and basophils by interacting with the chitobiose core of IgE glycans, it is speculated that higher intake of potato may increase the clinical symptoms as a result of non-allergic food hypersensitivity in atopic subjects.

The major proteins in garlic bulbs are alliinase and the low mol. wt. mannose-specific agglutinins or lectins ($ASA_{25}$). $ASA_{25}$ represents two agglutinins devoid of glycans: ASA I (a heterodimer), and ASA II (a homodimer). The agglutinins were purified from raw garlic extract (RGE) using a combination of gel filtration (Sephadex G-50) and cation exchange chromatography. Purified ASA I and II showed significant binding for the glycoproteins (HRP and avidin-alkaline phosphatase) compared to the raw extract, and their binding ability is comparable to that of the prototype mannose/glucose-specific lectin, concanavalin A (Con A). ASA II exhibited only one-third the specific hemagglutination activity of ASA I. These dietary lectins from garlic have been chosen for studying their effects on human leukocytes *in vitro* and mast cells *in vivo*. Both ASA I and ASA II were found to induce histamine release (HR) from leukocytes of atopic and non-atopic subjects. ASA I is more potent compared to ASA II, and HR by ASA I in the case of atopic subjects (61%) is significantly higher compared to that of non-atopic subjects (36%). Heat-processed garlic extract was found to retain ~70% of biological activity compared to RGE as assessed by hemagglutination and glycoprotein binding assays.

A major factor in non-allergic food hypersensitivity appears to be the interaction of dietary lectins with mast cells and basophils. Since IgE contains 10-12% carbohydrates, lectins can activate and degranulate these cells by cross-linking the glycans of cell-bound IgE. From the present study, it is clear that oligo-GlcNAc-and mannose-specific lectins (exemplified by potato and garlic lectins, respectively) interact with the glycans of non-specific IgE in inducing histamine release from mast cells and basophils. On the other hand, since IgE lacks GalNAc, horse gram lectin (GalNAc-specific) does not induce histamine release. Besides mannose and GlcNAc IgE glycans contain fucose, penultimate galactose, and terminal sialic acid residues. Therefore, it is expected that lectins specific for fucose, galactose, and sialic acid would, most likely, cause non-specific activation of IgE on basophils and mast cells.

The modulatory effects of the mannose-specific garlic lectins (ASA I and ASA II), and oligo-GlcNAc specific potato lectin was studied on lymphocytes in relation to mitogenicity. Both the garlic lectins were found to be mitogenic towards human peripheral blood lymphocytes (PBLs) and murine splenocytes as well as thymocytes. ASA I, ASA II, and RGE showed 3.5, 1.5, and 2.25 fold increase, respectively, in proliferation index as compared to control cells (human PBLs), as determined by MTT assay. ASA I and II displayed 4.5 and 3 fold increase, respectively, in the proliferation activity with murine splenocytes and thymocytes compared to the respective control cells. On the other hand, potato lectin, raw- and heat- processed potato extracts do not show any significant proliferation effect on both human PBLs, and murine splenocytes & thymocytes (<10% compared to Con A). ASA I and ASA II were unable to induce reactive oxygen species (ROS) from macrophages present in rat peritoneal exudate cells. Both potato and garlic lectins failed to induce the production of nitric oxide ($NO_2^-$) from human PBLs. From these observations, it is evident that garlic lectins possess mitogenicity for lymphocytes whereas potato lectin is devoid of the same.

Garlic lectins ASA I and II were tested for their ability to induce IgG response in BALB/c mice by intradermal and intranasal routes of administration. By intradermal administration, ASA I shows 4.2 fold increase, and ASA II shows 2.8 fold increase in IgG

response as compared to the reference protein ovalbumin (a poor antigen). Both lectins were found to be potent immunogens that indicates the systemic measure of immune response. By intranasal route, only ASA I (3.5 fold) shows a significant increase in IgG response than ASA II (1.2 fold) compared to the reference protein ovalbumin, which indicates the mucosal immune response. The anti-lectin IgG response for ASA I and ASA II is considerably significant as compared to the reference protein ovalbumin. During the experimental duration, there was no reduction in the body weight of animals, suggesting that garlic lectins are not growth-retardant. Based on the spleen and thymus weights, and their indices, there was a slight increase in the reference ovalbumin group and significant increases were seen in the lectin groups, indicating the activation of lymphoid organs, by these dietary lectins. Anti-lectin antibody response by both intradermal and intranasal routes of administration increased after each booster dose, and the titer was found to be very significant in the lectins group than in the reference ovalbumin group. These observations indicate that dietary garlic lectins are potent immunogens. The mucosal adjuvant potential of ASA I and II was investigated for a poor antigen ovalbumin, and only ASA I was found to have a marked adjuvant activity whereas ASA II does not. It appears that garlic lectins, are potent immunogens, in particular ASA I, have the potential for use as adjuvants in boosting the immune response of co-administered antigens such as vaccines since garlic lectins are derived from a dietary source and are not toxic.

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