

R - ACF-5468

INV.C.185.7/12/98

MANNOSE-BINDING LECTIN FROM SEEDS OF *ARTOCARPUS INTEGER*

A thesis presented for the

degree of

Master of Science

by

LIM SING BIN

Department of Biochemistry

Faculty of Medicine

University of Malaya

1997

Perpustakaan Universiti Malaya



A508308950

Dimikrofisikan pada..... 14-3-2000

No. Mikrofis..... IX520

Jumlah Mikrofis..... 3

HAMSIAH BT. MOHAMAD ZAHARI

UPR

UNIT REPROGRAFI
PERPUSTAKAAN UTAMA
UNIVERSITI MALAYA

ACKNOWLEDGEMENTS

This thesis and its supporting project could not have been completed without the support, attentive supervision and valuable guidance of my supervisor, Associate Professor Dr Onn Haji Hashim.

The generosity I encountered whilst working on the project was little short of astounding. My profound and sincere thanks go to Dr. Iskandar, Dr. Kanthimathi, Mr. Leong, Victor, Theng Theng, Rajes, Kak Azlila, Ada, Stephanie, Leong Joo, Yong, Anna, Jaya, Teo and Sharifah for their invaluable time, assistance and kindness. Not forgetting those who have offered their help and are hereby sincerely appreciated.

Tokens of appreciation to the wonderful, tireless staffs at the library of the Faculty of Medicine.

My respect and appreciation to both my beloved parents and family who gave so generously and naturally their loving support and affection.

Not a day goes by without my reflecting on my good fortune in having so many generous and loyal friends, many of whom provided both loving support and practical assistance.

Last but not least, I do hope that I have not left anyone out, and that if I have, they will forgive me, and accept my belated thanks.

CONTENTS

	PAGE
TITLE	i
ACKNOWLEDGMENTS	ii
CONTENTS	iii
ABBREVIATIONS	vii
LIST OF FIGURES	ix
LIST OF TABLES	xi
ABSTRAK (Bahasa Malaysia version)	xii
ABSTRACT (English version)	xiv
 INTRODUCTION	
1.1 History and definition of lectin	1
1.2 Occurrence and isolation	1
1.3 Classification of lectin	4
1.3.1 By structural homology	4
1.3.2 By sugar specificity	7
1.4 Biological roles of lectins	7
1.5 Applications of lectin	11
1.5.1 Blood typing and erythrocytes polyagglutinate studies	11
1.5.2 Fractionation of oligosaccharide or glycoproteins	12
1.5.3 Mitogenic stimulation of lymphocytes	15
1.5.4 Histochemical studies of normal and pathological conditions	16
1.5.5 Cell separation	17
1.6 Aims of investigations	17
 MATERIALS AND METHODS	
2.1 Artocarpus seeds	19
2.2 Mice	19
2.3 General materials	19
2.3.1 Fine chemicals	19
2.3.2 Cell culture materials	22

2.3.3	Radiochemicals	22
2.3.4	Normal human serum sample	23
2.3.5	Serological reagents	23
2.3.6	SDS-PAGE	23
2.3.7	Two dimensional electrophoresis	23
2.3.8	Chromatography support	23
2.4	Standard solution	24
2.4.1	Phosphate buffered saline	24
2.4.2	Citrate phosphate buffer	24
2.5	Methods	24
2.5.1	Determination of protein concentration	24
2.5.2	Preparation of crude extracts of champedak and jackfruit seeds	25
2.5.3	Preparation of sugar affinity columns	25
2.5.3.1	Activation of sepharose gel with divinylsulfone	25
2.5.3.2	Coupling of sugars to DVS-activated sepharose 4B	27
2.5.4	Purification of lectin	27
2.5.4.1	Purification of galactose-binding lectin	28
2.5.4.2	Purification of mannose-binding lectin	28
2.5.5	Preparation of lectin M-affinity column	29
2.5.6	Lectin M-Sepharose chromatography	29
2.5.7	Molecular weight determination by gel filtration chromatography	30
2.5.8	SDS-PAGE	30
2.5.9	Double diffusion	30
2.5.10	Competitive HRP-binding assay	32
2.5.11	Immunoelectrophoresis	33
2.5.11.1	Stock solution	33
2.5.11.2	Preparation of samples and electrophoresis	33
2.5.11.3	Staining and destaining the gels	34
2.5.12	Two dimensional electrophoresis	34
2.5.12.1	Standard solutions	34
2.5.12.2	Rehydration of immobiline dry strip	35
2.5.12.3	First dimensional run	35

2.5.12.4	Second dimensional run	36
2.5.13	Silver staining	37
2.5.13.1	Stock solutions	37
2.5.13.2	Staining procedure	38
2.5.14	Western Blotting	38
2.5.14.1	Stock solutions	38
2.5.14.2	Conjugation of lectin M to alkaline phosphatase	39
2.5.14.3	Protein transfer	40
2.5.14.4	Protein detection	41
2.5.14.5	Chromogenic visualisation of alkaline phosphatase	41
2.5.15	Mitogenic study	41
2.5.15.1	Tissue culture medium	41
2.5.15.2	Preparation of mitogen and sugars	43
2.5.15.3	Isolation of suspension cells from mouse organ	43
2.5.15.4	Preparation of murine B cells suspension	43
2.5.15.5	Preparation of murine T cells suspension	44
2.5.15.6	Cell counting	44
2.5.15.7	Determination of cell viability by trypan blue exclusion	44
2.5.15.8	Proliferation assay	45
2.5.15.9	Liquid scintillation counting	45
2.5.16	Effect of lectin on the secretion of immunoglobulins by B lymphocytes	45
2.5.16.1	Stimulation of Ig secretion by B lymphocytes using lectin	45
2.5.16.2	Antibody capture enzyme-linked immunosorbent assay (ELISA)	46

RESULTS

3.1	Isolation and purification of lectins	47
3.2	Structural study	47
3.2.1	Gel filtration chromatography on Sephadex G-100	47
3.2.2	SDS-polyacrylamide gel electrophoresis of the purified lectins	51
3.3	Binding specificity study	51

3.3.1	Determination of specificity for binding to glycoproteins	51
3.3.2	Effect of glycoproteins, sugars, and other substances on lectin M-HRP interaction	54
3.3.2.1	Effects of glycoproteins	54
3.3.2.2	Effects of sugars	56
3.3.2.3	Effects of metal ions and EDTA	56
3.4	Lectin M chromatography	56
3.5	Reactivity with human serum proteins	60
3.5.1	Immunoelectrophoresis analysis of interaction of lectin M with human serum proteins	63
3.5.2	Identification of lectin M reactive serum proteins	63
3.5.2.1	Analysis by two-dimensional electrophoresis	65
3.5.2.2	Western blotting	65
3.6	Biological activity of lectin M on murine lymphocytes	68
3.6.1	Effect on proliferation of murine thymocytes	68
3.6.2	Effect of mannose on lectin M stimulation	71
3.6.3	Effect on proliferation of murine B lymphocytes	71
3.6.4	Polyclonal activation of B cells for immunoglobulin secretion	74

DISCUSSION

4.1	Isolation and structural characterisation of champedak lectin M	77
4.2	Interaction of lectin M with selective glycoproteins	78
4.3	Effect of sugars and other substances on HRP-lectin M interaction	80
4.4	Binding specificity of lectin M-affinity column	82
4.5	Interaction of lectin M with human serum proteins	83
4.6	Mitogenic activity of lectin M	85
4.6.1	Effects on proliferation of murine T cells	85
4.6.2	Effects on proliferation of murine B cells	86
4.6.3	Polyclonal activation of B cells	87
4.7	Conclusion	87

REFERENCES

PUBLICATIONS	100
---------------------	-----

ABBREVIATIONS

The abbreviations used in this thesis are those recommended in the Instructions To Authors of the Biochemical Journal (1997), with the following additions:-

A ₂₈₀	- Absorbance readings at 280 nm
α	- Heavy chain of IgA
AP	- Alkaline phosphatase
B cells	- Cell lineage responsible for immunoglobulin production
C1	- Complement 1
Con A	- Concanavalin A
cpm	- Radioactive counts per minute
2D	- Two dimensional
DTT	- Dithiothreitol
DVS	- Divinylsulfone
FCS	- Foetal calf serum
γ	- Heavy chain of IgG
Gal	- D-galactopyranose
GalNAc	- N-acetylgalactosamine
GlcNAc	- N-acetylglucosamine
HEPES	- N-2 hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
Hr	- Hour
HRP	- Horseradish peroxidase
Ig	- Immunoglobulin
kDa	- Kilodalton
Man	- D-mannopyranose
M _r	- Relative molecular mass
μ	- Heavy chain of IgM
NeuNAc	- N-acetylneurameric acid
NHS	- Normal human serum

PBMC	- Peripheral blood mononuclear cells
PBS	- Phosphate buffered saline
PHA	- Phytohemagglutinin
SDS	- Sodium dodecyl sulphate
TBS	- Tris buffered saline
T cells	- Thymus derived lymphocytes
Tris	- Tris(hydroxymethyl)methylamine
Vh	- Volt-hours
WGA	- Wheat germ agglutinin

LIST OF FIGURES

Figures		Page
1.	General scheme for the isolation of lectins.	5
2.	Structural diversity of oligosaccharide of glycoproteins.	14
3.	<i>Artocarpus</i> fruits.	20
4.	Mice	21
5.	A representative protein standard curve as obtained by Protein Assay ESL Kit.	26
6.	Calibration curve for the determination of molecular weight using Sephadex G-100 gel filtration chromatography.	31
7.	Elution profiles of lectin isolated from mannose-Sepharose affinity chromatography.	49
8.	Gel filtration of mannose-binding lectin.	50
9.	SDS-polyacrylamide gel electrophoresis of champedak crude and purified lectins.	52
10.	Interactions of lectins with normal human serum, immunoglobulins and glycoproteins.	53
11.	Effects of glycoproteins on lectin M-HRP binding.	55
12.	Effects of sugars on lectin M-HRP binding.	57
13.	Effects of metal ions and EDTA on lectin M-HRP interaction.	58
14.	Elution profiles of N-linked oligosaccharide-containing glycoproteins on lectin M-Sepharose.	61
15.	Immunolectrophoresis analysis of lectin M interaction with normal human serum.	64
16.	Analysis of lectin-reactive serum proteins isolated by affinity chromatography.	66
17.	Western blotting analysis on lectin M-reactive human serum proteins.	67
18.	Proliferative response of murine thymocytes stimulated with lectins.	69

19.	Proliferative response of murine thymocytes stimulated with lectin C.	70
20	Agglutination of murine T cell.	72
21.	The effect of sugars on [³ H]-thymidine incorporation by thymocytes stimulated with lectin M.	73
22.	Nude mouse spleen cell proliferative response to lectin M.	75
23.	Proliferative response of purified murine T and B cell stimulated with lectin M.	76

LIST OF TABLES

Tables		Page
1.	A brief history of lectin research.	2
2.	Lectin families by structural homology.	6
3.	Lectins families by sugars specificity.	8
4.	Composition of RPMI-1640 medium.	42
5.	Total protein content and percentage recovery of mannose- and galactose-reactive lectins.	48
6.	Concentration of sugars and various substances that produced 50% inhibition on lectin M-HRP binding.	59

ABSTRAK

Lektin pengikat manosa, yang dikenali sebagai lektin M champedak, telah diasingkan dari ekstrak mentah biji cempedak (*Artocarpus integer*) dengan menggunakan kromatografi keafinan. Apabila kromatografi penurasan gel dilakukan, lektin ini dielusikan dalam satu puncak yang bersamaan dengan berat molekul 64 kDa. Electroforesis gel SDS-poliakrilamida menunjukkan bahawa lektin pengikat manosa ini terdiri daripada beberapa polipeptida 16.8 kDa. Sesetengah daripada polipeptida ini wujud sebagai dimer melalui ikatan disulfida.

Apabila diuji dengan semua jenis iso (isotype) imunoglobulin, lektin M champedak ini menunjukkan interaksi selektif yang kuat dengan IgE dan IgM manusia, dan interaksi lemah dengan IgA2. Interaksi pengikatan lektin M ini adalah tidak bersandar kepada ion-ion logam. Lektin tersebut juga didapati berinteraksi dengan horseradish peroksidase, ovalbumin, tiroglobulin (khinzir), α_1 -asid glikoprotein, transferin dan α_1 -antitripsin (manusia) seperti diperlihatkan melalui eseai pengikat HRP. Ia mengikat lebih kuat pada ligan $\text{Man}\alpha 1\text{-}3\text{Man}$ berbanding dengan $\text{Man}\alpha 1\text{-}2\text{Man}$ dan $\text{Man}\alpha 1\text{-}6\text{Man}$. Profil elusi kromatografi keafinan lektin M dengan pelbagai jenis glikoprotein dengan struktur karbohidrat yang diketahui menunjukkan bahawa pengikatan lektin mengutamakan oligosakarida jenis kompleks biantenari dan sesetengah oligosakarida jenis kandungan tinggi manosa.

Interaksi di antara lektin M dengan protein-protein serum manusia telah dikaji dengan menggunakan elektroforesis 2-dimensi dan blot afiniti lektin M. Lektin M didapati berinteraksi dengan beberapa protein yang mengandungi rantai glikan

pengikat-N seperti α_1 -asid glikoprotein, transferin dan α_1 -antitripsiin, IgM dan IgA dalam bentuk natif dan juga dengan rantai berat- γ IgG dalam keadaan terturun.

Kajian yang dilakukan untuk melihat kesan lektin M ke atas limfosit tikus telah menunjukkan ia merangsang proliferasi sel T dengan kepekatan optimum 2.5 $\mu\text{g/ml}$ bila dikultur selama 3 hari. Lektin M dipercayai adalah mitogen yang khusus kepada sel T kerana ia tidak mengaruh sintesis DNA apabila dikultur dengan sel-sel limpa tikus *mude* (iaitu strain tikus tanpa timus). Ia juga tidak berupaya untuk mengaruh sel B rehat untuk membeza kepada sel plasma yang merembeskan imunoglobulin.

ABSTRACT

A mannose-binding lectin, termed champedak lectin-M, was isolated from the crude extract of the seeds of champedak (*Artocarpus integer*) by affinity chromatography. On gel filtration chromatography, the lectin eluted in a single peak at elution volumes corresponding to 64 kDa. SDS-PAGE showed the mannose-binding lectin to be composed of 16.8 kDa polypeptides with some of the polypeptides being disulphide-linked to give dimers.

When tested with all isotypes of immunoglobulins, champedak lectin-M demonstrated a selective strong interaction with human IgE and IgM, and a weak interaction with IgA2. The binding interactions of lectin-M were metal ion independent. The lectin was also shown to interact with horseradish peroxidase, ovalbumin, porcine thyroglobulin, human α_1 -acid glycoprotein, transferrin and α_1 -antitrypsin as observed in HRP-binding assay. It demonstrated a binding preference to Man α 1-3Man ligands in comparison to Man α 1-6Man or Man α 1-2Man. The elution profile of various glycoproteins with defined carbohydrate structure on lectin M-affinity chromatography have demonstrated that lectin M preferentially bind to biantennary complex-type and certain high-mannose-type oligosaccharides.

The interaction of lectin M with human serum proteins was studied using 2D-electrophoresis and lectin M-affinoblotting. Interestingly, lectin M interacted with quite a number of N-linked glycan chain containing proteins such as haptoglobin, α_1 -acid glycoprotein, transferrin, α_1 -antitrypsin, IgM, and IgA in their native conformation as well as the γ -heavy chain of IgG under reducing condition.

Investigation on the effect of lectin M on murine lymphocytes revealed that it stimulated the proliferation of murine T cells with an optimal concentration of 2.5 µg/ml in a 3 days culture. Lectin M is believed to be a T-cell mitogen as it does not induce significant DNA-synthesis when cultured with spleen cells from nude mouse. It was also incapable of inducing the resting B cells to differentiate into an immunoglobulin secreting plasma cells.