PURIFICATION AND CHARACTERIZATION OF A LECTIN FROM TAMARILLO FRUITS (*CYPHOMANDRA BETACEA*)

by

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A thesis presented in partial fulfilment of requirements for the degree of Doctor of Philosophy in Biotechnology at Massey University, Palmerston North, New Zealand

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Lectins specific in their binding to oligomers of β1,4 linked N-acetylglucosamine were identified in the fruits of *Cyphomandra* species of the family Solanaceae. Thus, *Cyphomandra* species can be considered as a new source of lectins for basic and applied studies.

New lectins (designated as CBL1 and CBL2) were identified from tamarillo fruits (*Cyphomandra betacea*). CBL1 was purified. Biochemical characterization, subcellular localization and molecular sequence analysis for this new lectin were made. CBL2, which was immunologically unrelated to CBL1, was not further characaterized.

CBL1 could be readily purified using affinity and ion exchange chromatography. CBL1 comprised two subunits joined by nonconvalant interactions. Subunit size was 25 kDa. N,N,'N",N""-tetraacetylchitotetraose was the most effective carbohydrate for inhibition of CBL1 induced agglutination of rabbit erythrocytes. CBL1 consists of abundant residues of Cys (16 %), Gly (14 %), Glx (13 %), Ser (11 %), Pro (9 %) and Asx (7 %), and to a lesser extent, hydroxyproline residues.

CBL1 was found to be an abundant, extremely stable and developmentally regulated protein. It was found predominantly in cell walls of fruit tissues using immunofluorescence techniques. CBL1 could play a defence role in seed development.

Despite the general resemblance of chemical composition and carbohydrate specificities, no cross-reaction among solanaceous lectins in double immunodiffusion tests performed

in gels containing their carbohydrate ligands was demonstrated, suggesting they may not have similar epitopes.

Four tryptic peptides and the N-terminal fragment of CBL1 were sequenced, which showed some homologies with the Gramineae lectins. Since CBL1 and the Gramineae lectins shared similar properties such as amino acid composition and sugar specificities, it is suggested that CBL1, a solanaceous lectin, might be evolutionarily related to the Gramineae lectins.

Two cDNA clones were isolated with anti-CBL1 serum, and sequenced. One of them (X200), which reacted weakly with anti-CBL1 serum, was 96 % identical with a bacterial gene *ilv*C encoding acetohydroxy acid isomeroreductase. The peptide encoded by this cDNA could have some similar epitopes to CBL1, which resulted in its isolation. Another clone (X208), which showed stronger reaction with anti-CBL1 serum, was found to contain putative peptide sequences which did not show homology with CBL1 peptide sequences. This clone could be derived from one domain of CBL1's coding region, while the peptide sequences could be confined to another domain. Complexity in immunoscreening the clone encoding CBL1 is discussed, and future work on the isolation of cDNA clone encoding this interesting lectin is suggested.

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ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bisacrylamide	N,N'-Methylene-bis-acrylamide
BpB	Bromophenol blue
BSA	Bovine serum albumin
CBL1	tamarillo lectin (*Cyphomandra betacea*), subuint size 25 kDa
CBL2	tamarillo lectin (*Cyphomandra betacea*), subuint size larger than 50 kDa
cDNA	complementary DNA
Con A	concanavalin A
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulphoxide
dNTPs	2'-Deoxyribonucleoside 5'-triphosphates
dCTP	2'-Deoxycytidine 5'-triphosphate
DSA	*Datura* seed lectin (thorn apple lectin, TAL)
ds DNA	double stranded DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Fuc	fucose
Gal	galactose
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
Glu	glucose
Hepes	N-2-hydroxy ethyl piperazine-N'-2-ethane sulphonic acid
HPLC	High-pressure liquid chromatography
IEF	isoeletric focusing
IPTG	isopropylthio-β-D-galactoside
LEL	tomato lectin (*Lycopersicon esculentum*)
LB	Luria broth
Man	mannose
NBT	nitro blue tetrazolium chloride
NeuNAc	sialic acid
PBS	phosphate-buffered saline
PBSB	phosphate buffered saline containing bovine serum albumin
pfu	plaque forming unit
PHA	phytohemagglutinin
SDS	sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophresis
SM	Phage buffer supplemented with 0.1 % gelatin

STE	Tris Cl buffered NaCl/ethylenediaminetetraacetic acid
STL	lectin of potato tuber (*Solanum tuberosum*)
TAL	thorn apple lectin (*Datura* seed lectin, DSA)
TBE	Tris-borate/EDTA electrophoresis buffer
TCA	trichloroacetic acid
TE	Tris buffered ethylenediaminetetraacetic acid
TEMED	N,N,N′,N,-tetramethylethylene diamine
TFA	Trifluoroacetic acid
TNT	Tris-Cl containing NaCl and Tween-20
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
Tris	Tris(hydroxymethyl)aminomethane
TTBS	Tris-Cl/tween-20 and NaCl buffer
WGA	Wheat germ agglutinin
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1 LITERATURE REVIEW

1.1 Historical background of lectin research

It is generally accepted that a doctoral thesis published in 1888 by Stillmark marked the beginning of lectin research (Kocourek, 1986; Sharon and Lis, 1987). While investigating the constituents of seeds of some plants belonging to the Euphorbiaceae family, in 1888 and 1889, Stillmark made the startling observation that a partially purified toxic proteinaceous preparation from castor beans (*Ricinus communis*) that he called ricin agglutinated erythrocytes. Stillmark also observed agglutination of liver cells, epithelial cells and leukocytes by ricin. His work started a series of theses and papers. One of these works by Hellium showed that a toxic extract of jequirity (*Abrus precatorius*) also caused red cells to clump together. The new agglutinin was named abrin.

By using these two toxins, Ehrlich could establish some of the fundamental concepts of immunology (Kocourek, 1986). For example, rabbits fed with small amounts of jequirity seeds developed a certain degree of immunity against abrin. Immunity could be increased by additional parenteral administration of the toxic protein. He was able to show the specificity of the proteins (i.e. antibody) found in serum of animals after administration of abrin and ricin. The anti-abrin serum could neutralize the toxic activity of abrin but not that of ricin and vice versa, and these toxins could also be specifically precipitated by proteins found in serum in response to the administration of these toxins. Ehrlich also demonstrated a quantitative relationship between the amount of antiserum and the amount of toxin that could be neutralized by it, and he also showed that during pregnancy

immunity to the toxins was transferred from mother to the offspring in the blood and that after birth it may be passed through the milk.

In 1909, Landsteiner and Raubitschek (Kocourek, 1986) reported for the first time the presence of non-toxic lectin in the seeds of the leguminosae family, such as in beans (*Phaseolus vulgaris*), pea (*Pisum sativum*), lentil (*Lens culinaris*) and vetch (*Vicia sativa*). They identified these plant lectins as proteins and showed that they were water soluble, non-dialysable, insoluble in alcohol, thermolabile, could be salted out by electrolytes and gave positive biuret and xanthoprotein reaction. They also observed that porcine gastric mucin caused "deagglutination" of erythrocytes agglutinated by ricin, abrin or bean extracts. Although the authors was unable to explain it, this report showed for the first time the inhibition of haemagglutination by carbohydrate substances present in the mucin.

Following the work by Landsteiner and Raubitschek (Kocourek, 1986), search for haemagglutinins revealed that they existed predominantly in seeds of the Leguminosae, Euphorbiaceae and Solanaceae families. There were also discoveries of preferential localization of lectin in certain seed structures and various organs of plants, e.g. potato tubers (Kocourek, 1986). Lectins were also found in a wide variety of other categories of organisms in the early part of this century (Kococurek, 1986): in fungal fruiting body of *Amanita mascara* and *A. solitaria;* in bacteria such as *Staphyloccus aureus, Vibrio, E.coli;* in chicken plague virus and animal such as the horseshoe crab (*Limulus polyphemus*), the American lobster (*Homarus americanus*) and in snake venoms.

From 1919 to 1934, there was no significant progress in

lectin research except the work about Con A by Sumner (1919). Obviously, there was no driving force for theoretical and practical uses of lectins and in addition, their physiological function was unknown and their further exploration was restricted by the methodology and knowledge of protein biochemistry at that time.

After the period 1919-1934, there were many significant discoveries about lectins by the second half of the 1960s, such as isolation of concanavalin A, blood specificity, mitogenic properties, specific binding of malignant cells and specific recognition of carbohydrate structures by lectins.

Sumner (1919) was able to obtain two crystallisable components from jack bean (*Canavalia ensiformis*), one of which, concanavalin B, could be dissolved in 10% NaCl and the other, called concanavalin A (Con A) was soluble in high concentrated salt. Con A was found to be the agglutinating constituent and in later times was to become a widely used lectin. Thus Con A was the first lectin to be isolated in pure form. In 1936, Sumner and Howell reported that in addition to its ability to agglutinate cells such as red blood cells and yeasts, Con A also precipitated glycogen, starch granules and mucoprotein in solution. They demonstrated that haemagglutination by Con A was inhibited by cane sugar and suggested that this might be due to the reaction of this protein with carbohydrates on the surface of the erythrocytes. This was believed to be the first report on the nature of haemagglutination and sugar specificity of lectins. Sumner and Howell (1936a) also accomplished the characterization of the lectin. These authors (1936b) also demonstrated that the activity of Con A required divalent metal ions.

The studies by Sugishita in 1935 and Jonsson in 1944 on eel serum agglutinins and later by Boyd in 1947 and Renkonen in 1948 on plant agglutinins have been considered as the first approach to the practical application of blood group specific lectins and, in general, to the investigation of lectins as recognition molecules (Kocourek, 1986). Sugishita reported two types of agglutinins in the Japanese eel *Anguilla japonica*, one was nonspecific towards the ABO system and the other showed a high titre against O type cells. Jonsson reported a similar agglutinin from the eel *Anguilla anguilla* was specific towards group O cells, he then suggested this agglutinin could be employed as an anti-O(H) blood typing reagent. Lectin was then considered as a special type of antibody. It took almost 30 years before its non-immunoglobulin nature was proven (Bezkorovainy *et al.*,1971). Boyd observed that an extract from dried lima beans (*Phaseolus lunatus* sym.*limensis*) "agglutinated erythrocytes of some human individuals, but those of others only weakly if at all". He then realized that these results correlated with blood groups. Renkonen in 1948 undertook systematic study of the agglutinating properties of extracts of seeds from the Leguminosae family. He found that six of them showed affinity either for A or O erythrocytes. Boyd and Reguera in 1949 found about half of the 70 varieties of *Phaseolus lunatus* seeds showed high anti-A titre. These works followed by others stimulated new interest by haematologists and immunologists and provided the first practical uses of lectins which caused a new wave of searching for novel lectins for this type of work (Kocourek, 1986).

The blood type-specific lectins played a central part in elucidating the chemical nature for ABO blood group specificity. Watkins and Morgan (1952) demonstrated that the agglutination of group A blood cells by the lima bean lectin was most readily inhibited by α linked N-acetylglucosamine

and that of O-type cells by the lectin of *Lotus tetragonolobus* was best inhibited by α-linked L-fucose. They suggested that α-N-acetylglucosamine and α-L-Fucose were the sugar determinants of A and O blood group respectively. This work also provided the evidence of sugar presence on cell surfaces.

In 1957, Mäkelä divided lectin-reactive monosaccharides into four categories based on their configuration at C-3 and C-4 positions of the pyranose form of hexose as shown Fig.1-1. L-fucose-binding lectins are specific to Mäkelä's group I, galactose- and N-acetylgalactosamine-specific lectins to group II, and mannose- and/or glucose-specific lectins to group III. Lectins binding to the Mäkelä's group IV have not been found yet (Wu *et al.*, 1988). Based on this classification, group specificity also applies to N-acetylglucosamine- and sialic acid-specific lectins, and to the complex sugar (e.g. chitin) binding lectins.

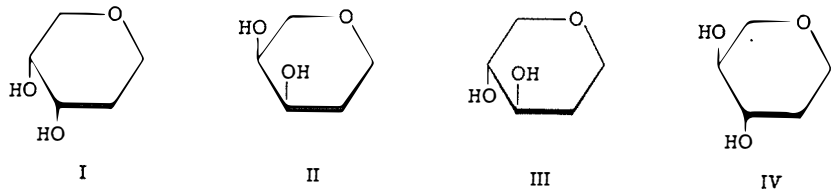


Fig.1-1 Classification of pyranose of lectin-reactive monosaccharides (Mäkelä, 1957).

In 1960, Nowell made a very exiting discovery with the observation that lymphocytes, which were then believed to be a class of dead-end cells at their terminal stage of development and incapable of further division, could be triggered into an unsuppressed state of active growth and proliferation *in vitro* by the phytohaemagglutinin (PHA) from *Phaseolus vulgaris* seeds. This discovery led to a number of important immunological investigations and also led to

discoveries of various other lectins which could also stimulate lymphocyte division (some are specific for lymphocytes from certain animals). Today, Nowell's discovery greatly facilitates the examination of chromosomes in human beings and certain animals and has led to the expansion of human cytology and the increased understanding of relationships between chromosome abnormalities and diseases. The stimulation of lymphocyte division by lectins also provides a probe for better understanding of the mechanisms about how a signal from the cell surface can be transmitted into the nucleus to trigger DNA replication and subsequent cell division.

In 1963, Aub and his collaborators made an important observation that a lipase-containing extract from wheat germ (*Triticum vulgare*) exhibited the ability to inhibit tumour growth and cause agglutination of several transformed cell lines but did not appear to agglutinate their normal counterparts. Later, Burger and Goldberg (1967) separated the agglutinating activity from the lipase activity and identified the substance responsible as wheat germ agglutinin (WGA). It was this important discovery that attracted many scientists to study the lectins in the hope that lectins might provide an important tool to study the role of the cell surface in cancer cells and thus the riddle of cancer in general. Three years later, Sela and co-workers found that the soybean lectin could also distinguish normal and malignant cells and some other lectins were later found to have the same property. Although it is now clear that not all lectins possess this property, all these works provided evidence that malignant cells display different cell surface from that of normal cells.

The activities of lectins such as blood-type specificity, mitogenicity and their ability to distinguish malignant and

normal cells had led to a search for new lectins and studies on their biochemistry in the 1950s and the early 1960s. However there were no convenient and efficient procedures for isolating lectins. Conventional protein purification methods were effective but time consuming. By the end of 1964, there were only five lectins which were isolated and partially characterized. They were: Con A (Sumner and Howell,1936a.b), the castor bean lectin (Takahashi *et al.*, 1962; Ishiguro *et al.*,1964 a,b) , soybean lectin (Palansch and Liener, 1953; Wada *et al.*, 1958), lectin from kidney bean (Rigas and Osgood,1955), lectin from blackbean (Jaffé and Gaede, 1959). It was Agrawal and Goldstein in 1965 who introduced affinity chromatography into lectin research, which greatly facilitated simple and efficient isolation of lectins. They (1965) demonstrated that cross-linked dextran (Sephadex) could be employed as a specific absorbent for Con A. After washing Sephadex 200 with 1 M NaCl solution, non-interacting components of protein extract passed through the column and the specifically-absorbed Con A could be eluted by 0.1 M D-glucose in the same solution. Although the principle of affinity isolation of lectins could theoretically apply to any lectin, due to the limited number of appropriate carriers available that time, conventional chromatographic procedures were still widely used in the 60s and the early 70s.

During the 70s, the search for new lectins showed some decline and its focus of interest was shifted from plants to other taxonomic phyla including vertebrates. However due largely to the ease of isolating lectins by affinity chromatography and other advanced purification procedures, the most important area of research was the systematic studies of the molecular properties of individual lectins. Ranging from main physico-chemical parameters, amino acid composition and amino acid sequence to establishing three-dimensional structure, these data provided a fundamental basis for understanding of the lectin activities at the

molecular level. Equally important were the extensive structural studies of lectin receptors. All these studies together would be expected to give insight to *in vivo* function of lectins.

During the 1980s, the advances of DNA technology had a sweeping impact on lectin research. Numerous structures of lectins were elucidated by sequencing their genes or cDNA. Structures were then compared by computing methods to search for sequence homology. This trend of research still continues to the present time and without any doubt it will continue to flourish in this decade.

In summary, lectins, which were first discovered a century ago, later were found to exist in almost every category of living organisms. Although their endogenous cellular functions are not clear, their theoretical and practical application have been the driving force behind the extensive and intensive researches of lectins.

1.1.1 Definition of lectins

The definition of lectins has undergone several changes since their first discovery. Lectins were also referred to as agglutinins, phytohaemagglutinins, phytoagglutinins and protectins.

The term lectin (Latin *legere*: to choose) was first coined by Boyd and Shapleigh (1954a,b) to refer to a group of plant agglutinins, some of which were human blood group specific. With the discovery of carbohydrate-binding proteins in diverse categories of organisms, the term lectin has been

broadened to include sugar binding proteins from any source (Aswhell, 1977). Goldstein et al.(1980) proposed the definition that a lectin is a sugar-binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates. The Nomenclature Committee of the International Union of Biochemistry (Dixon, 1981) has recommended this definition with small modifications, e.g. the deletion of the term glycoprotein. It should be pointed out that this definition was based on their *in vitro* biological properties, which was an operational one. It implied that a lectin should have at least two sugar-binding sites or more. Thus, this definition would exclude those transport proteins, hormones and toxins (ricin, abrin etc.), which might be evolutionarily related to lectins as defined by Goldstein *et al.*(1981). It should be pointed out here that no purified lectins have been shown to exhibit enzymatic activity. Although it was reported that some lectins possess glycosidase activity, when examined critically, it proved to be that some glycosidases under certain conditions acted as lectins (Shannon, 1983; Goldstein and Poretz, 1986).

Kocourek and Horějšé (1981) proposed a less restrictive definition of lectins as sugar-binding proteins or glycoproteins of non-immune origin which are devoid of enzymatic activity towards sugars to which they bind and do not require free glycosidic hydroxyl groups on these sugars for their binding. This definition would include those toxins which contain one sugar-binding site.

With increasing structural and functional examination of lectins, it was determined that many lectins could also contain a second type of binding site that interacted with a non-carbohydrate ligand, and that there were structural homologies between bivalent lectins (defined by Goldstein *et*

al.,1980) and related proteins such as those with one carbohydrate-binding site. These findings suggested that both our conceptualization of lectin function and the formal definition of this class of lectins have been changed. Thus, Barondes (1988) proposed to define lectins as carbohydrate-binding proteins other than an enzyme or antibody. This rather broad definition would help to focus our attention on the evolution of lectins and on their adaptation for a variety of functions in biological systems. This definition has been used in this thesis.

1.2 General overview of plant lectins

1.2.1 Introduction

Lectins exist not only in plant but also in other categories of organisms such as animals (reviews for animal lectins: Sharon and Lis, 1989; Barondes, 1984; Paroutaud *et al.*, 1987; Drickamer, 1989). However, plants still serve as the main source of lectins. Reviewed here is a classification of plant lectins based on carbohydrate binding properties, the structural properties of plant lectins and possible functions of plant lectins *in vivo*. Applications of plant lectins are also summarized.

1.2.2 Classification

The classification of plant lectins discussed here is solely based on their known carbohydrate binding specificity, thus is rather arbitrary, and might not reflect their *in vivo* functions. However, such a basis for classification is useful for the practical applications of plant lectins.

The specificity of a lectin is usually defined in terms of the monosaccharides that best inhibit lectin-induced animal cell (red blood cells) agglutinations (Goldstein and Poretz, 1986). Some lectins however do not agglutinate red blood cells (e.g. white clover lectin was found only to agglutinate its symbiont *rhizobium*), and some lectin-induced reactions cannot be inhibited by monosaccharides, but instead are inhibited by oligosaccharides (e.g. solanaceous lectins). In the author's opinion, lectin specificity would best be classified in terms of the carbohydrate that best inhibits the lectin-induced cell agglutination or carbohydrate precipitation.

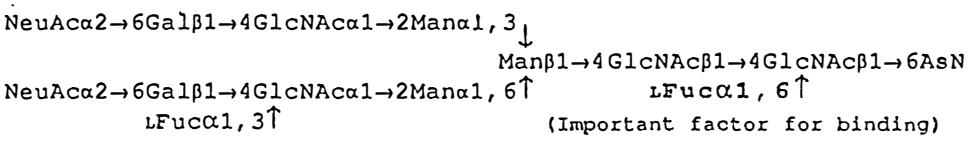
As discussed previously (Section 1.1), Mäkelä (1957) suggested that lectin-reactive monosaccharides could be divided into four classes based on their configuration at C-3 and C-4 of the pyranose ring (Fig.1-1). In the past decade, detailed biochemical characterization of lectins has given enormous information of their carbohydrate specificity, which has led to the classification of the plant lectin-reactive saccharides into the following classes (Goldstein and Poretz,1986; Wu *et al.*,1988): I. N-acetylglucosamine; II. N-acetylgalactosamine; III. glucose/mannose; IV. galactose; V. L-fucose; VI. sialic acid. A compilation of the plant lectins based on their reactivity towards the six groups of saccharides is shown in Appendix.

Lectins differ markedly with respect to their anomeric specificity. Some, for example, Con A (Smith and Goldstein, 1967) and lectins from *G. simplicifolia* (Hayes and Goldstein, 1974) and *L. tetragonolobus* (Pereira and Kabat,1974) exhibited pronounced specificities for the α anomers of mannose or glucose, galactose, and lactose, respectively. However, other lectins such as soybean agglutinin (Lis *et*

al.,1970; Pereira *et al.*, 1974) and *R.Communis* agglutinin (RCA_{II}) (Nicolson *et al.*, 1974) were found to be almost lacking in anomeric specificity.

Although the binding sites of some lectins appear to be complementary to a single glycosyl unit, many others have been shown to possess large binding sites which may accommodate two or up to six sugar residues. For example, WGA and solanaceous lectins interact most strongly with β-(1,4)-linked N-acetylglucosamine oligomers. The peanut lectin binds to Galβ1,3GalNAc units (Wu, 1984) and *Wisteria floribunda* mitogen binds preferentially the $Man_3GlcNAc$ core region of the asparagine-linked glycoprotein (Goldstein and Poretz, 1986).

Interestingly, mannose-specific lectins from *Lens culinaris* and *Pisum sativum* recognized a glycopeptide of the following structure with L-Fucose attached to the non-reducing end of GlcNAc:



Without the α(1,6) linked L-fucose, these lectins would bind to this glycoconjugate less by 10 to 17 fold (Debray *et al.*, 1981). However, whether the L-fucose is also involved in the binding site(s) of these lectins is not clear. It is likely that L-fucose might influence the structure of the conformation of this glycoconjugate so that it would better fit the binding sites of these lectins than those without the attached L-Fucose.

It is also interesting to note that lectin of *A. bisporus* (Presant and Korfeld, 1972) and the blood group N-specific lectin of *Vicia graminea* (Lisowaska and Duk, 1975) appeared to recognize carbohydrate sequences together with the amino acid or peptide to which the latter are linked. The exact mechanism involved is not clear.

Many lectins tolerate some variations at the C-2 position of the sugars which they bind. For example, Con A (Goldstein *et al.*,1965; So and Goldstein, 1967) and the broad bean lectin (Allen *et al.*, 1976) exhibit a primary specificity for mannose, but also bind to glucose and, to a lesser extent, N-acetylglucosamine. However, hydroxyls of C-3 and C-4 of sugars that lectins bind are very critical, because lectins can tolerate very little variation at these sites. For example, mannose/glucose-binding lectins (Mäkelä,1957; Goldstein *et al.*,1965; Allen *et al.*, 1976) did not interact with galactose and vice versa. Similarly, N-acetylglucosamine binding lectins did not interact with N-acetylgalactosamine (Allen *et al.*, 1973). The physico-chemical basis for this is not understood. It might imply that these two groups (mannose and galactose specific lectins) of lectins have followed separate evolution lines resulting in the selectivity on C-3 and C-4 of the sugars to which they bind.

Our understanding of the nature of carbohydrate-lectin interaction is still at its early stage. Roberts and Goldstein (1984a) reported that an apparent entropy ($\Delta S^{\circ\prime}$= -4.9 cal deg^{-1} mol^{-1}) of lima bean lectin interaction with L-Fucose on the blood group A trisaccharide is more than that of its N-acetylgalactosamine binding (-10.2 cal deg^{-1} mol^{-1}). Since the enthalpies of binding for both ligands are similar, this indicates that the large (40 fold) difference in affinities of the two ligands is due primarily to the difference in entropy of binding. Thus, in this case, lectin-

carbohydrate interaction is driven at least partially by a hydrophobic force. On the basis of thermodynamic parameters in the wheat germ agglutinin-sialyoligosaccharide interactions, Kronis and Carver (1985) concluded that the dominant forces stabilizing the associated complex would appear to be intermolecular hydrogen bonds and van der Waals forces. Based on the evidence of ^{1}H-NMR measurements, semi-empirical energy calculation and interactive graphics modelling, Hamodrakas *et al.*(1989) proposed that hydrophobic interactions together with van der Waals interaction and hydrogen bonds contribute to the stability of the complex of 4'-nitrophenyl-α-D-mannopyranoside and Con A. However, high-resolution x-ray crystallographic analysis of carbohydrate-lectin complexes would be needed to better understand the exact nature of carbohydrate-lectin interaction.

1.2.3 Chemical and structural properties

1.2.3.1 Introduction

In order to better understand the *in vitro* properties and functional implications of lectins *in vivo*, knowledge of their chemical and structural properties is of paramount importance. Examples for each major class of lectins described in Section 1.2.2 will be discussed below (For detailed reviews: Lis and Sharon, 1981; Goldstein and Poretz, 1986).

1.2.3.2 N-acetylglucosamine-specific lectins

As shown in Appendix, N-acetylglucosamine-specific lectins comprise a diverse group of lectins which exhibit primary

specificity for GlcNAc and/or its β(1,4)-linked oligomers, and in some instances, glucosamine. Lectins from several families are represented here: Gramineae (Peumans *et al.*, 1983; Miller and Bowles, 1983; Lord, 1985; Stinissen *et al.*, 1983;), Solanaceae (Section 1.3), Cucurbitaceae and Leguminosae (*Griffonia simplicifolia* II, *Cytisus sessilifolis,* and *Ulex europeus* II). The Man/Glu-specific lectins (e.g. jack bean, pea, lentil, fava bean, and the genus *Vicia* agglutinins) also bind N-acetylglucosamine but only weakly and only if the sugar occurs in α-anomeric linkage at chain ends of oligosaccharides (Goldstein and Poretz, 1986).

Sequence homologies have been observed in this class of lectins and some related proteins. For example, sequences of WGA, rice lectin, root-specific barley lectin, nettle lectin, chitinase, and some wound-induced genes (*win*) from potato have shown to be homologous (Fig.1-2) (Lucas *et al.*, 1985; Chapot *et al.*, 1986; Stanford *et al.*, 1989; Lerner and Raikhel, 1989; Wilkins and Raikhel, 1989). However, more sequence information is needed for the understanding of whether this class of lectins originated from a single source or from separate ones.

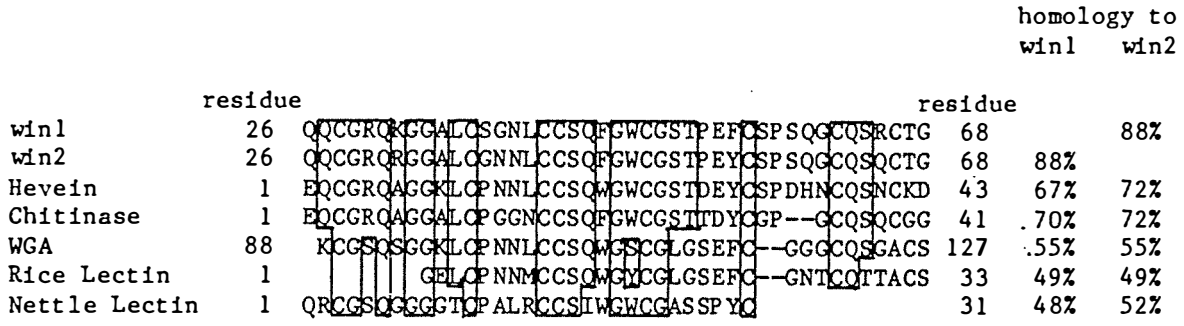


Fig. 1-2 Homologies among the deduced amino acid sequences of *win*1 and *win*2 and protein sequences of hevein, chitinase, wheat germ agglutinin, rice lectin and nettle lectin (Stanford *et al.*, 1989).

1.2.3.2.1 Wheat germ agglutinin (WGA, *Triticum vulgare* agglutinin)

WGA, is the most thoroughly studied member in this class of lectins. WGA is a dimeric and carbohydrate free protein consisting of two identical subunits. Molecular weight was 21 kDa with 171 amino acids per polypeptide chain (Wright, 1984). This lectin was isolated as a mixture of three or four molecular species (isolectins) that differ in their electrophoretic mobility (Allen *et al.*,1973; Rice and Etzler, 1975). The four isolectins were distributed as follows: 35% of I, 50% of II_a, 5% of II_b and 10% III. (Rice and Etzler, 1975). Amino acid compositions of these isolectins were nearly identical, being abundant in glycine residues (25%) and cysteine residues (20%), although form I contained no histidine residues whereas the other three forms contain two histidine residues per subunit. Occurrence of these multiforms of WGA is due to the genome ploidy -- each of four sets of genomes directs synthesis of a single subunit or polypeptide species (Peumans *et al.*, 1982a).

The x-ray crystallographic analysis of WGA was completed at 1.8 Å resolution (Wright, 1987). The protein crystallizes in space group C2 with two protomers (I,II) per asymmetric unit, Each protomer consists of four structurally homologous and spatially distinct domains (A,B,C,D) (Fig.1-3)

The carbohydrate-binding specificity of WGA has been studied by a variety of techniques, such as hapten inhibition of specific precipitation of glycoconjugates, changes in fluorescence of the lectins or of chromatogenic ligands, equilibrium dialysis, NMR and x-ray diffraction. Several important features of the carbohydrate-binding properties can be summarized as follows (Goldstein and Poretz, 1986):

1. Sugar specificity:
   N,N′,N,"N′",N""-pentaacetylchitopentose >
   N,N′,N",N"′-tetraacetylchitotetraose >
   N,N′,N"-triacetylchitotriose >> N,N′-
   Diacetylglucosamine >>N-acetylglucosamine
   (WGA also binds to N-acetylneuraminic acid)

2. For N,N′-Diacetylglucosamine, there were two binding locations per protomer (four per dimer): a primary location and a less well defined secondary location. Thus, each dimer of WGA binds to four molecules of N,N′-Acetylglucosamine. For N-Acetylneuraminic acid, the binding site in the protomer is at the primary location, thus each dimer of WGA binds two molecules of N-Acetylneuraminic acid (Wright,1980a). A schematic illustration of these binding sites is shown in Fig.1-4.

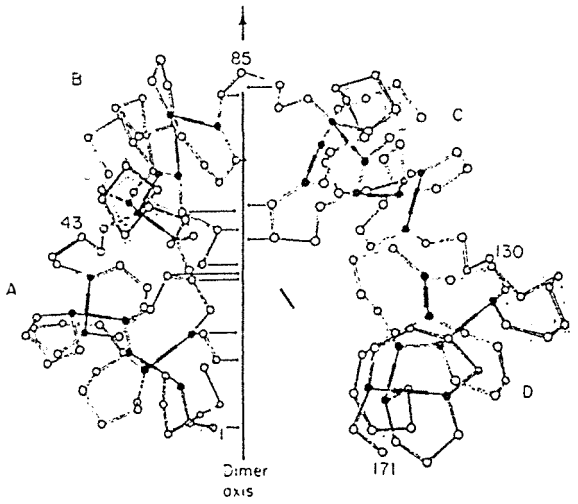


Fig. 1-3 α-Carbon backbone drawing of the WGA protomer. Structural domains were labelled as A,B,C and D. The position of all S-S bridges are shown by heavy bars. The position of the dimer axis has been placed to illustrate the proximity of residues in domain A and B to this axis. Residues involved in protomer-protomer contact across the dimer axis are marked by horizontal arrows (Wright, 1987).

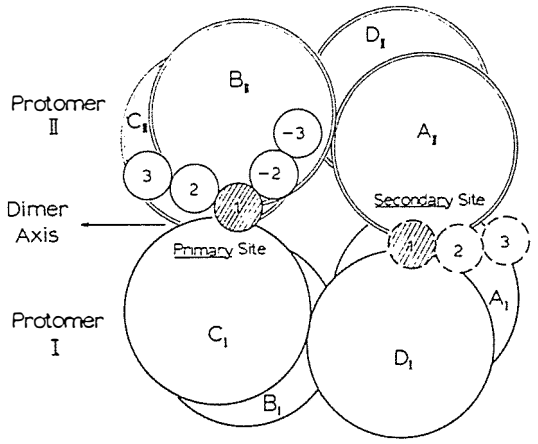


Fig.1-4 Schematic illustration of the disposition of the primary and secondary binding locations on the WGA dimer. The domains of protomer I are labelled A_I,B_I,C_I and D_I and those of protomer II A_{II},B_{II}, C_{II} and D_{II}. Each unique binding location is subdivided into subsites (small circles). GlcNAc oligomers bind at subsites 1, 2 and 3 in both binding locations, whereas NeuNAc oligosaccharides utilize subsites 1, -2 and -3 in the primary binding location only. Subsite is shaded to indicate strongest binding localization (Wright, 1980a).

The data on whether other GlcNAc-specific lectins have similar three-dimensional structural properties as WGA are not available.

1.2.3.3 N-acetylgalactosamine/galactose-specific lectins

These lectins are the most diverse groups in their taxonomical classification, yet their sequences show strong homology (Goldstein and Poretz, 1986). However the structural characteristics shared by these lectins seem to be related more to the origin of the plants than to carbohydrate-binding specificity of these proteins, for example, soybean lectin

showed extensive homology with mannose/glucose specific lectins, since the latter and soybean lectin are all from the family Leguminosae. Most lectins of these two groups contain Ca^{2+} and/or Mn^{2+}, although lectins from *Bauhinia purpurea, Maclura pomifera, Ricinus communis* and *Abrus precatorius* lack bound metal. Galactose-specific lectins also include toxins such as *Ricinus communis* (Euphorbiaceae), *Abrus precatorius* (Leguminosae), *Viscum album* (Loranthaceae) and *Adenia digitata* (Passifloraceae). Discussed here are *Glycine max* lectin (GalNAc-specific) and *Phaseolus vulgaris* lectin (Gal-specific lectin).

1.2.3.3.1. *Glycine max* lectin (Soybean agglutinin)

The molecular weight of soybean agglutinin is 120 Kda (Lotan *et al.*, 1974; Fountain and Yang, 1977), it comprises four subunits of molecular weight close to 30 kDa. The amino acid composition analysis showed that it was comparatively rich in acidic and hydroxylic amino acids and devoid of cysteine. A complete amino acid sequence of soybean agglutinin was also determined which was derived from the nucleotide sequence of *Le1* DNA gene (Vodkin *et al.*, 1983). The mature protein sequence is preceded by a hydrophobic signal sequence of 32 amino acid sequence. Soybean lectin is a glycoprotein containing 7% by weight carbohydrate consisting of mannose and N-acetylglucosamine (Lis and Sharon, 1978). Soybean lectin is a metalprotein containing Ca^{2+} and Mn^{2+} (Jaffe *et al.*, 1977), and contains one high affinity site for Mn^{2+}, and four transition metal sites. Preliminary x-ray crystal structure analysis showed the crystals to be monoclinic, space group C2 with the unit cell containing one tetramer (Shannon *et al.*, 1984).

Carbohydrate specificity-binding studies on the soybean

lectin showed that it exhibits greater affinity for N-acetylgalactosamine, its glycosides and oligosaccharides in which N-acetylgalactosamine was the non-reducing terminal unit. Galactose and its derivatives were less reactive. Its carbohydrate binding affinity is as follows (Debray *et al.*,1981):

GalNAcα or β1→linked GalNAcα1,3Galβ1,3GlcNAc and GalNAcα1,3Gal >>
GalNAcα1,3[$_L$Fucα1,2]Galβ1,4GlcNAcβ1,6R, Galα1,6Glc (melibiose), Galβ1,6Glc, Raffinose, Stachyose, Galβ1,4Glc (lactose) and $_L$Fucα1,2Galβ1,4GlcNAcβ1,6R > Gal.

Soybean lectin possesses four carbohydrate binding sites per tetramer (De Broeck, 1984). In addition, a hydrophobic binding site near the carbohydrate binding site on soybean agglutinin was also present, which was demonstrated by thermodynamic measurements of the association constant of N-dansylgalactosamine (De Broeck *et al.*,1984) , which binds to soybean agglutinin about 20 times stronger than N-acetylgalactosamine. The lectin agglutinates mouse spleen B cells and not T cells, and has been used for the separation of the two classes of lymphocytes (Reisner *et al.*, 1976). It does not agglutinate normal hamster, human, rat, mouse cell lines, however it agglutinates transformed counterparts of the cell lines from mouse, human and rat.

1.2.3.3.2. *Phaseolus vulgaris* lectin (PHA, Red kidney bean lectin)

This lectin has several fascinating properties such as lymphocyte mitogenicity (Nowell, 1960, for a discussion see Section 1.1.1) and different binding affinity towards leukocytes and erythrocytes (Goldstein and Poretz, 1986). PHA

has five isolectins which consist of two different subunits of L and E(R) (Fig.1-5). The L and E subunits bind leukocytes, and erythrocytes respectively.

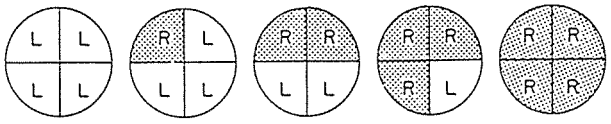


Fig. 1-5 Schematic representation of the tetrameric structure of the five isolectins from *Phaseolus vulgaris* (Miller *et al.*,1975).

Leukoagglutinin (L-PHA, L_4) consists of four identical subunits with serine as the N-terminal amino acids and PI = 5.25, exhibits strong affinity for lymphocytes but weak affinity for erythrocytes (Miller *et al.*, 1973, 1975). L_4 is a potent lymphocyte mitogen. The haemagglutonin (H-PHA, E_4) contains four identical E subunits with alanine as N-terminal amino acid and PI = 5.95 (Miller *et al.*, 1973). This form also exhibits a strong affinity for erythrocytes (Yachnin *et al.*, 1972). The three intermediate forms are tetramers that contain various proportions of L and E subunits (LE_3, L_2E_2, L_3E_1). Native isolectins could be dissociated in 6 M guanidinium chloride and reconstituted to the original mixture by removal of these denaturing reagents (Felsted *et al.*, 1977; Monsigny *et al.*, 1978b). These hybrid species can cause a mitogenic response of lymphocytes proportional to their content of L subunits. However, E_4 also possesses strong mitogenic activity of its own (Glad and Borrebaeck, 1984). They showed that E_4 binds 14 glycoproteins in the serum used for mitogenic study whereas L_4 only binds 9 glycoproteins, and thus suggested some of the glycoproteins masked mitogenic activity of the E_4 isolectins.

L and E subunits have identical molecular weights (Miller *et al.*, 1973, 1975), differing in their N-terminal amino acid sequence (1-7), but are identical in positions 8-24 and in their three C-terminal residues (Miller *et al.*, 1975). The carbohydrate composition of the E and L subunits is identical (Miller *et al.*, 1975). The molecular weight of L4 is 126 kDa with that of L subunit 31 Kda. L4 contains no sulphur-containing amino acids, but a high proportion of aspartic acid, leucine, serine, threonine and valine, and mannose and glucosamine. Mn^{2+} and Ca^{2+} are both essential for their biological activity.

Carbohydrates specific to either E_4 or L_4 are complex ones. Their minimum structural unit of carbohydrate specific for E_4 is as follows (Yamashita *et al.*, 1983):

Galβ1,4GlcNAcβ1,2Manα1,6↓
GlcNAcβ1,4Manβ1,4GlcNAcβ1,4R_3
R_1→GlcNAcβ1,2Manα1,3↑
R_3

R_1 and R_2 represent either hydrogen atoms or sugars, R_3 represents either GlcNAc or (Fucα1,6)GlcNac.

For L_4, the specific carbohydrates are as follows:

Tri- and Tetra-antennary oligosaccharides containing Galβ1,4GlcNAc-linked units at the non-reducing end >>
Penta-2,6^4 > Tri-2,6^4 >
Galβ1,4GlcNAcβ1,2Manα1,3↓
Man (hepta-)>
Galβ1,4GlcNAcβ1,2Manα1,6↑
Galβ1,4GlcNAcβ1,2Man > GlcNAcβ1,2Man.

Whether PHA has binding sites other than those of ion and carbohydrate is not clear.

1.2.3.4 Mannose/glucose-binding Lectins

This group of lectins comprises a group of agglutinins found in the family Leguminosae, often in high concentration. They represent a most thoroughly studied group of lectins. Among them are concanavalin A (Con A) from Jack bean (*Canavalin ensiformis*), lectins from the pea (*Pisum sativum*), the lentil (*Lens culinaris*), the fava bean (*Vicia faba*), the common vetch (*Vicia cracca*) and sainfoin (*Onobrychis viciifolia*).

Almost all lectins in this class are composed of two light (α) chains and two heavy (β) chains having general structure $\alpha_2\beta_2$. Exceptions to this structure are Con A (four identical subunits) and the sainfoin lectin (two identical subunit). Non-covalent interactions are involved in the subunit binding, one of the exceptions to this is lima bean lectin which has been reported to contain a disulphide bond between the subunits (Goldstein and Poretz, 1986). Metal ions, e.g. Mn^{2+} and/or Ca^{2+}, are required for their activity, which binding sites are conserved among these lectins (Strosberg *et al.*, 1986). These lectins are rich in acidic and hydroxylic amino acids, but devoid or low in sulphur-containing amino acids. All members of this class of lectins are mitogenic for lymphocytes (Goldstein and Poretz, 1986).

The amino acid sequences of many lectins in this class of lectins have been reported, revealing extensive homology among themselves (Fig.1-6). Leguminous lectins which belong to other classes of sugar specificity such as soybean lectin also show homology to this class of lectins. All leguminous lectins are initially synthesized as single polypeptide chains of a molecular weight of around 30 kDa, which may be post-synthetically cleaved into a larger β and a smaller α subunits after removal of a 20-residue hydrophobic leader

sequence. Variable degrees of glycosylation account for the dispersion of molecular weights as well as for most of the apparent polymorphisms of these lectins due to the fact that Asn-X-Thr/Ser, a characteristic carbohydrate attaching site is not well conserved in this class of lectins. The amino acid residues which constitute the sugar binding sites are also not well conserved (Strosberg *et al.*,1986). This may be reflected by the differences in saccharide-affinities exhibited by leguminous lectins (Hořejší *et al.*, 1977; Goldstein and Heyes, 1978).

When compared to the three-dimensional structure of Con A, these lectins in this group have been revealed to have similar structural features by computational methods (Olsen, 1983). In fact, several lectins from this group have been crystallized, and their three-dimensional structures have been determined, showing homology to Con A (Riskulov *et al.*, 1984; Reeke and Becker, 1986; Meehan *et al.*,1982; Mande *et al.*,1988).

One of the most interesting properties of this group of lectins is the conservation of their hydrophobic cavities. All the residues which contribute to the three dimensional structure of the well characterized hydrophobic cavity of Con A are found in their homologous positions or have some of them replaced by chemically similar amino acids (Strosberg *et al.*, 1986). Although it is not known what substances bind to this cavity *in vivo*, *in vitro* studies showed that the plant hormone auxin could be bound to the cavity of Con A (Edelman and Wang, 1978), albeit with weak affinity (K_a=7 x 10^2M^{-1}). Moreover, several other lectins have been shown to exhibit high affinity for adenine, a phytohormone (K_d = 1.2 x 10^{-5}M for lima bean lectin; K_d= 2.0 x 10^{-6}M for *Dolichos biflorus* lectin; K_d= 1.3 x 10^{-5}M for soybean lectin) (Roberts and Goldstein, 1983b). These observations suggest a role of

lectins *in vivo* as a hormone carrier or receptor.

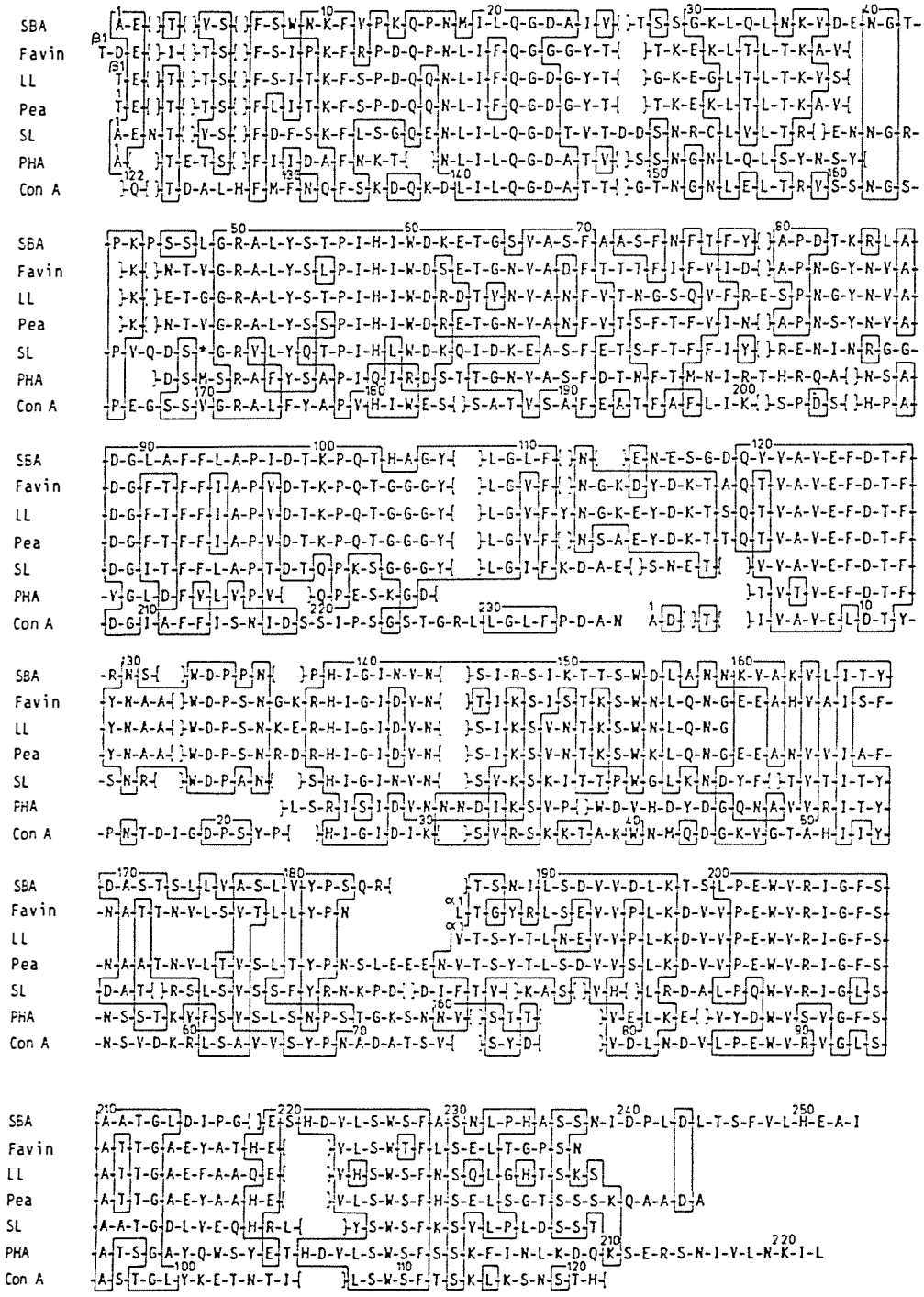


Fig. 1-6 Complete sequences of soybean agglutinin (SBA), fava bean lectin (favin), lentil lectin (LL), pea lectin, sainfoin seed lectin, phytohaemagglutinin (PHA), and Concanavalin A (con A). The identical residues have been boxed in. Empty brackets correspond to deletions. The empty space at the end of the LL β and the favin as well as αchains denote posttranslational removal of small fragments (Strosberg *et al.*, 1986).

1.2.3.4.1 Concanavalin A (Con A) (*Canavalia ensiformis*, Jack Bean)

Con A comprises four (at pH 7) subunits which are carbohydrate free. The molecular weight of its subunit is 26.5 kDa. At pH 5.6 or below, Con A occurs as a dimer, and above pH 5.6, Con A occurs as a tetramer. The aggregation of Con A was also found to be temperature-dependent. Each subunit of Con A contains one Ca^{2+}, and one Mn^{2+} ion, which appeared to be essential for the carbohydrate binding of Con A (Sumner and Howell, 1936b; Agrawal and Goldstein, 1968).

The three dimensional structure of Con A has been determined by x-ray crystallographic methods (Edelman *et al.*, 1972; Hardman and Ainsworth, 1972). The schematic representation of the three dimensional structure of Con A is shown in Fig. 1-7.

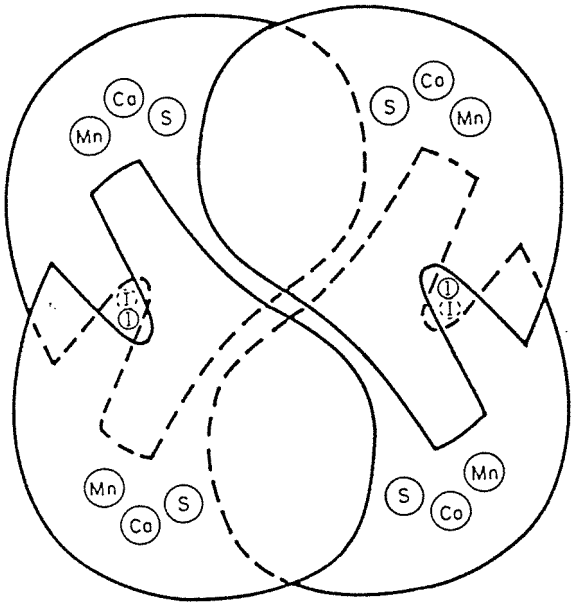


Fig.1-7 Schematic representation of Con A tetramer. Ca, Mn, and S indicate the positions of the Ca^{2+}, Mn^{2+}, and carbohydrate binding sites respectively. I indicates the position of the hydrophobic binding site present in crystals of I222 spacing group (Becker *et al.*,1976).

Mannose, in its α-anomeric form, shows the greatest affinity for the Con A sugar binding site. However, methyl α-mannoside is about 7 times as potent as mannose, and α(1,2) linked mannose oligosaccharides the di- and tri-saccharides, are 4 and 20 times as potent as methyl α-mannoside. Other polysaccharides containing the non-reducing terminal α-linked glucose, mannose, or N-acetylglucosamine groups were found to be reactive to Con A. A recent review on carbohydrate binding properties of Con A is available (Goldstein and Poretz, 1986).

As already described above, Con A was found to contain hydrophobic cavities or binding sites, one of which seemed *in vitro* binding to auxin, a plant growth hormone. So far, about three classes of the hydrophobic sites have been identified [a detailed discussion on this topic can be found in the review by Goldstein and Poretz (1986)]. The exact role(s) of these hydrophobic binding sites remains to be elucidated.

1.2.3.5. L-Fucose specific lectins and sialic acid specific lectins

Detailed biochemical properties of these two groups of lectins are less understood than for other groups of lectins. Only a few lectins of this group have been reported, and only WGA, the sialic acid binding lectin from wheat germ, has been studied in detail (discussed in Section 1.2.3.2). The reason for the rare occurrence of these lectins in plant kingdom is not clear.

1.2.3.6 Summary

In summary, lectins from different classes based on their carbohydrate specificities have been discussed. Members of some classes of lectins have been shown to be related to each other with respect to their protein sequences, structures and properties. Lectins have not only carbohydrate binding sites, but also contain other binding sites such as metal binding sites and hydrophobic binding sites of unknown function.

1.2.4 Possible *in vivo* functions and applications of plant lectins

1.2.4.1 Possible *in vivo* functions of plant lectins

Although lectins were discovered a century ago, their roles in the Plant Kingdom are still largely unknown. A number of suggestions for their roles have been proposed.

Trifoliin, a lectin specific to 2-deoxy-D-glucose from white clover (*Trifolium repens*) roots, binds specifically to its symbiont *Rhizobium trifolii* (Dazzo *et al.*, 1978). Antibodies to this lectin bind mostly to the surface of the roots of white clover. This lectin could be released from roots by 2-deoxyglucose. These data suggest that trifoliin acts as a bridge between similar carbohydrates on both surfaces of clover root and its symbiont rhizobia. The involvement of lectin in the *Rhizobium*-legume symbiosis was further demonstrated (Díaz *et al.*, 1989). *Rhizobium leguminosarum* bv. *viciae* nodulates the cross group of pea, vetch and lentil, whereas *R. leguminosarum* bv. *trifolii* nodulates white clover only. After the pea lectin gene was cloned to an expression

vector and introduced into the roots of white clover, it was found that the transgenic roots could be nodulated by *R. leguminosarum* bv. *viciae*.

Lectins may also play defence roles in plants. For instance, the lectin from stinging nettle rhizome inhibited growth of several pathogenic fungi which contain chitin as a cell wall component, but in a different mode to the common antifungal chitinase (Broekaert *et al.*, 1989). The nature of the inhibitory effect on growth of cell walls is thus thought to be the chitin binding properties of this lectin. However, other chitin-binding lectins such as rice lectin, WGA, and potato lectin have not been demonstrated to show inhibitory effects on the growth of chitin-containing fungi, possibly due to their large sizes which might render them difficult to gain access to active sites involved in cell wall growth. The products derived from the two wound-induced genes *win*1 and *win*2 in potato tubers were found to be homologous to WGA, rice lectin and the lectin from nettle (Stanford *et al.*, 1989) (Fig.1-2). It is possible that *win*1 and *win*2 are members of a lectin gene family from potato, since potato lectin can also be induced by wounding (Casalongué and Pont Lezica, 1985), the potato lectin pattern can be altered by virus X (Scheggia *et al.*, 1988). Biochemical characterization of their gene products is necessary to study their role during mechanical wounding.

N-terminal sequences of five glycoforms of α-amylase inhibitor from the seeds of common bean (*Phaseolus vulgaris*) were revealed to have two different N-terminal sequences, and these two sequences (11 and 15 amino acids long) match the two stretches of lectin-like protein (LLP) deduced from lectin gene from the seeds of the same plant (Moreno and Chrispeels, 1989). This lectin gene is 65% and 45% homologous to a major lectin from the seed (PHA) at the nucleotide and

amino acid sequence respectively. In addition, the amino acid composition of α-amylase inhibitor was shown to resemble that of LLP. Furthermore, the molecular size of the precursor was shown to have the same size as LLP, and anti-α-amylase serum was cross-reactive with the LLP gene product expressed in *E. coli*. Thus it was concluded that the LLP gene or one very close to it encoded this α-amylase inhibitor (Moreno and Chrispeels, 1989). It is known that α-amylase inhibitor inhibits insect and mammalian α-amylase, but not plant α-amylase. Thus, LLP could provide a defence role. However, it is not known that the defence role requires the carbohydrate-binding property of the lectin or lectin-like proteins discussed above.

The gene of arcelin, another seed protein from wild bean (*Phaseolus vulgaris*), might have evolved from a phytohaemagglutinin gene or genes (Osborn *et al.*,1988). Arcelin is a defence protein which has toxic effects on a major bean bruchid pest *Zabrotes subfasciatus*.

A number of other possible roles of lectins such as pathogenesis, self-incompatibility, cell wall elongation, enzymes, carbohydrate catchers and transporters have also been proposed (For a recent review: Etzler, 1986). However, more experimental evidence is required to support such role(s) and they are not discussed here.

1.2.4.2 Applications of lectins

Lectins are widely used as tools for the study of glycoconjugates both in solution or on the cell surfaces. A brief compilation of the lectin application is shown below (Table 1-1). More detailed discussion can be found in several

reviews (Lis and Sharon, 1986a,b; Sharon and Lis, 1987)

Table 1-1. Major uses of lectins (Lis and Sharon, 1986a; Broekaert *et al.*, 1989)

1. Isolation, purification, and structural studies of carbohydrate-containing polymers.
2. Investigation of complex carbohydrate structures on surfaces of animal cells, bacteria, virus, and subcellular particles.
3. Investigation of the architecture of cell surfaces and its changes upon malignant transformation.
4. Blood typing, structural studies of blood group substances; identification of new blood types; diagnosis of secretors.
5. Isolation of lymphocyte subpopulations and of a stem cell-enriched fraction of bone marrow suitable for transplantation.
6. Studies of the genetics, biosynthesis, and function of cell-surface glycoconjugates.
7. Mitogenic stimulation of lymphocytes; studies of events occurring upon initiation of cell division; studies on lymphokines; studies of chromosomal constitution of cells and detection of chromosomal abnormalities.
8. Studies of specific carbohydrate binding sites on proteins.
9. Biological control of pathogens.

1.3 Solanaceous Lectins

Solanaceous lectins are less extensively studied than those from Leguminosae, Euphorbiaceae and Gramineae. Potato lectin (STL, *Solanum tuberosum*), tomato lectin (LEL, *Lycopersicon esculentum*) and *Datura* seed lectin (DSA or TAL, *Datura stramonium*) are the only ones from the Solanaceae family which have been characterized biochemically. Their characteristics are summarized as follows.

1.3.1 Chemical composition and structure

Tomato lectin, LEL, contains about 50% carbohydrate, of which 85% is L-arabinose and 15% D-galactose (Nachbar *et al.*, 1980). It occurs as a single glycosylated polypeptide with M_r of 71 kDa (Nachbar *et al.*, 1980). However, it has been recently shown that M_r value for LEL is about 130 kDa (Merkle and Cummings, 1987; Zhu and Laine, 1990).

Datura seed agglutinin, DSA, is composed of two nonidentical subunits [M_r 46 kDa (subunit A) and 40 kDa (subunit B)] joined by disulphide bonds (Kilpatrick *et al.*, 1978; Crowley and Goldstein, 1981). It contains 93-94% L-arabinose and 6-7% D-galactose. The L-arabinose occurs as short, un-branched arabinofuranosyl units (containing β1,2- and β1,3-linkages) covalently bound to hydroxyproline (Desai *et al.*, 1981; Ashford *et al.*,1982a). Galactose occurs both as single α linked substituents and as α1,3-linked disaccharide units bound to serine. DSA can be separated into three individual lectins by hydrophobic interaction chromatography on phenyl-sepharose, two of them are homodimers (AA and BB) and the third one is AB (Broekaert *et al.*, 1987). M_r values for A and B are 32,000 and 28,000 respectively, smaller than those reported earlier. The A-subunit has a higher affinity for N-acetylglucosamine oligomers than the B-subunit.

Potato lectin, STL, is a dimeric glycoprotein (Monomer M_r 50 kDa) containing about 50% carbohydrate, of which 94% is L-arabinose and 6% D-galactose (Allen and Neuberger, 1973). Glycosylation of the lectin is limited to the one domain which contains all the hydroxyproline. This glycosylated domain has mono-. di-, tri- and tetra-saccharides of arabinofuranose linked to hydroxyproline and single galactopyranose residues linked to serine. The tri-

arabinoside contained only β1,2-linkages between sugars but the tetra-arabinoside contained a terminal α1,3-linkage in addition to the β1,2-linkages. The other domain of this molecule was found to contain all of the cysteines as well as the carbohydrate binding sites (Allen *et al.*, 1978; Ashford *et al.*, 1982a; Allen, 1983). A hypothetical model for the structural model of potato lectin is shown in fig. 1-8.

Treatment of STL with trifluoromethanesulfonic acid resulted in a de-glycosylated protein with an amino acid composition identical to the native lectin (Desai *et al.*,1983). The de-glycosylated lectin retained its haemagglutonating activity.

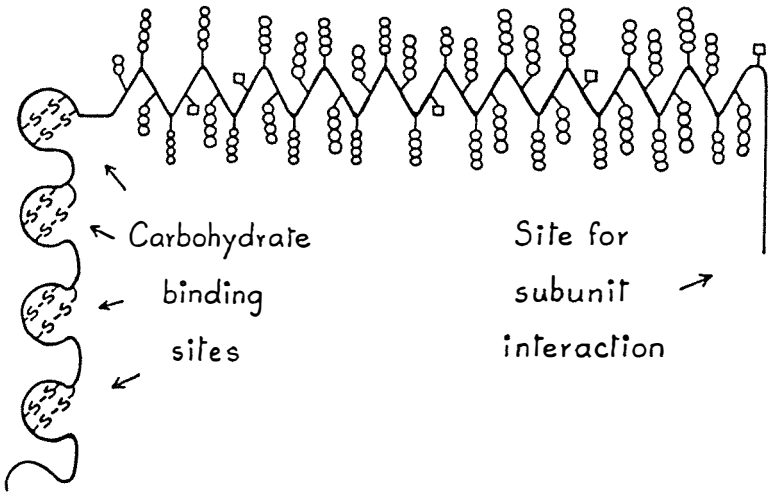


Fig. 1-8 Hypothetical model of the structure of potato lectin. O = Arabinofuranoside; □ = galactopyranoside (Allen, 1983).

Solanaceous lectins generally contain about 50-60% protein and similar amino acid composition. They are especially rich in the amount of residues of hydroxyproline, serine,

cysteine, and glycine. The detailed amino acid compositions of these lectins are given in Table 3-1 of this thesis.

Since the sequences of solanaceous lectins are not yet available, it is not known whether these lectins show structural homology. The overall similarity in their chemical compositions and carbohydrate binding properties could suggest that they are a group of closely related proteins. Lectin extracts of *Datura* seed, tomato seed, potato seed, *Nicandra* seed, *Capsicum* seed, tomato fruit juice, potato fruit juice, potato tuber were all reactive with anti-DSA serum (Kilpatrick *et al.*, 1980). Lectins from *Datura stramonum* seeds and tomato fruit juice also reacted with anti-STL serum, however not with de-arabinosylated STL glycopeptide (Ashford *et al.*, 1982a). This immunological data suggests that the cross reaction among solanaceous lectins results from the reaction of antiserum with the arabino-part of these lectins. Thus the arabino-parts of these lectins might share a similar structure. However, the interpretation of these data should be made with caution, since it does not rule out the possibility that the cross reaction of antiserum with other solanaceous lectins was simply from the carbohydrate binding properties of these lectins. This is a point taken up in more detail in Chapter 4 of this thesis.

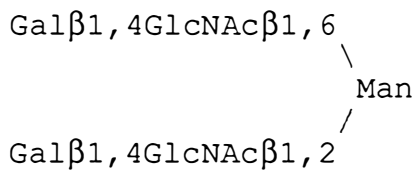
Circular dichroism analysis indicated that potato lectin contains about 35% polyproline II conformation (i.e. a left-handed helix with three residues per turn and a pitch of 9.36 Å), 34% type II β-turn and 31% irregular conformation. The polyproline II conformation is stabilized by oligosaccharides of this lectin, and the type II β-turn is stabilized by disulphide bridges of this lectin (Van Holst *et al.*, 1986).

Other hydroxyproline-rich glycoproteins of plant origin show

the presence of the polyproline II conformation (Homer and Roberts, 1979; Van Holst and Fincher, 1984; Van Holst and Varner, 1984). For example, extensin, also as rich in arabinosylated hydroxyproline as STL, is entirely in the polyproline II conformation and is stabilized by oligosaccharide chains. For STL the polyproline II conformation is located in the serine/hydroxyproline-rich domain. It has been suggested that this domain was derived from a portion of the plant cell wall hydroxyproline-rich glycoprotein (HRGP) gene which fused a gene encoding a cysteine/glycine rich protein (Showalter and Varner, 1986).

1.3.2. Carbohydrate specificities

Solanaceous lectins are chitin binding proteins which bind to β1,4-linked oligomers of N-acetylglucosamine. To a degree, the longer the chain, the more stable the lectin-carbohydrate complex will be. The tetramer of N-acetylglucosamine was the best saccharide to interact with LEL, STL and DSA (Nachbar *et al.*, 1980; Allen and Neuberger, 1973; Desai *et al.*, 1981;). It was later found however that the best inhibitor of DSA was the following penta-saccharide (Crowley *et al.*, 1984):



N-acetylglucosamine has little inhibitory effect on the haemagglutination induced by this group of lectins. But, some animal glycoproteins such as fetuin, asialofetuin, ovomucoid, glcophorin and other glycopeptides have profound inhibitory effects on the lectin-induced haemagglutination (Nachbar *et al.*, 1980; Debray *et al.*, 1981). Immobilized DSA and LEL are able to bind repeating N-acetyllactosamine sequences in asparagine-linked oligosaccaharides (Cummings and Kornfeld,

1984; Merkle and Cummings, 1987b; Zhu and Laine, 1989; Callaghan *et al.*, 1990). STL also binds the repeating N-acetyllactosamine sequences (Callaghan *et al.*, 1990). The binding constants of the lectin-carbohydrate interaction are given in Table 1-2.

Table 1-2. Binding constants (K_a) of interaction of solanaceous lectins with β1,4-linked oligomers of N-acetylglucosamine (β1,4-linked) (x 10^3 M^{-1}).

Carbohydrates	DSA	STL	LEL
Dimer	na	2.0[a]	na
Trimer	3.75[b]	0.9x10[c]	na
Tetramer	8.7[d]	3.5x10[c]	na

[a] Matsumoto *et al.*,1980, 1982; Ashford *et al.*, 1982b
[b] Crowley, 1982
[c] Ashford *et al.*, 1982b
[d] Crowley *et al.*, 1984
na--not available

1.3.3. Distribution, localization, and possible function of solanaceous lectins.

Since solanaceous lectins have not been studied systematically, their distribution within plant taxa is not yet clear. DSA is associated with cell walls in the seed coat and epidermis (Broekaert *et al.*, 1988). Subcellular fractionation studies indicated that STL is tightly, but non-covalently, bound to the plant cell wall (Casalongué and Pont Lezica, 1985). LEL is located predominantly in the locular fluid of ripe tomatoes (Merkle and Cummings, 1987a), suggesting tomato lectin may be an extracellular protein. Lectins from potato seem to play a defence role as discussed in Section 1.2.4.1. However, systematic research at the molecular and cellular level on the solanaceous lectins is

essential in order to understand their roles in the plant.

1.4 Aim of this study

The objective of this study was to isolate and characterize a new solanaceous lectin from fruits of red tamarillo (*Cyphomandra betacea*) following detection of agglutination activity in extracts during a preliminary screening of economically significant fruits available in New Zealand. Characterization of the new lectin designated CBL1 has been performed by biochemical and molecular biological techniques. A study of the immunological relationship of CBL1 to other solanaceous lectins and of its subcellular localization within fruit tissues is also described.

CHAPTER 2 MATERIALS AND METHODS

2.1 Fine chemicals, lectins and enzymes

The following materials were purchased from Sigma chemical Company, USA: tomato lectin (LEL), potato lectin (STL) and *Datura* seed lectin (TAL), TPCK treated trypsin (Type XIII, EC 3.4.21.4), protein molecular weight standards for protein gel electrophoresis[bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trysinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa)]. The following materials were purchased from BDH, England: Folin-Ciocalteau reagent, protein molecular weight standards for gel filtration column, carrier ampholytes ('Resolyte' pH 3.5-10). Isoelectric focusing standards [phycocyanin (pI 4.65), β-lactoglobulin B (pI 5.1), bovine carbonic anhydrase (pI 6.0), human carbonic anhydrase (pI 6.5), equine myoglobin A (pI 7.0), human hemoglobin A (pI 7.1), human hemoglobin C (pI 7.5), lentil lectin (3 bands) (pI 7.8, 8.0, and 8.2), cytochrome C (pI 9.6)], a silver staining kit, western blotting components and gold total protein staining reagent were bought from Bio-Rad, USA. Phytohaemagglutinin (PHA) was purchased from Wellcome, England. Radioactive materials (^{35}S-methionine, ^{35}S-ATP and ^{32}P-ATP) were purchased from New England Bio-Lab, USA. Donkey anti-rabbit IgG and Phycoerythrin-streptavidin cytochemical staining kit were purchased from Amersham, UK. Some restriction enzymes, cloning vector, cDNA synthesis, *in vitro* translation kits, T7 DNA polymerase sequencing system, *Exo*III enzyme were purchased from Promega, USA. Some restriction enzymes, and goat anti-rabbit IgG conjugated with alkaline phosphatase were purchased from BRL, USA. Anti-LEL serum was a kind gift from Dr D. Kilpatrick. S-Sepharose, Sephacryl S-200, Sephadex G75 and oligo-dT cellulose were purchased from Pharmacia,

Sweden. Other chemicals are of highest quality available from various commercial suppliers.

Saccharides used for the inhibition test: L(+)-arabinose, D(+)-galactose, inulin, lactose and sucrose were purchased from BDH; cellobiose and α-D(+)melibiose (United States Biochemical Corporation, USA); D(+)xylose was from (Serva); N-acetylglucosamine, N,N'-diacetylchitobiose, N,N',N"-triaetylchitotriose, N,N',N",N"'-tetraacetylchitotetraose, N-acetyllactosamine, maltose, L-sorbose, fructose, D-glucose, D(+)mannose, 2-deoxy-D-Glucose, 1-O-methyl-α-D-glucopyranoid, N-acetylglucosamine, D(+)trehalose, rhamnose, melezitose, arbutin, esculin hydrate, salicin, D-fucose, L-fucose, ribose, sialic acid, methyl-α-D-galactopyranoside, methyl-α-D-glucopyranoside, and methyl-α-D-mannopyranoside (Sigma Chemical Company, USA); arabino xylan wheat flour, carboxylmethylcellulose, dextran, araban, carob bean G-lactomannan, arabino galactan, corn cob xylan, starch, polygalacturonic acid, laminarin (β1,3 glucan), lichenin, coffee bean β1,4-mannan, yeast mannan, nigeran (from *A.niger*), pectin, gentiobiose, α-D-sophorose, arabic acid and fucoidin (kindly provided by Dr.I.G.Andrew, Chemistry and Biochemistry Department, Massey University).

2.2 Vectors, bacterial strains and growth conditions

Bacteria strains and vectors are summarized in Table 2.1. *E. coli* was grown in Luria Broth (LB) (1 % w/v NaCl, 0.5 % w/v yeast extract and 1 % v/w bacto-tryptone) at 37°C with shaking. *E. coli* used for transfection was grown in LB medium supplemented with 0.2 % (w/v) maltose and 20 mM $MgSO_4$, or supplemented with appropriate antibiotics when used for plasmid propagation. For plates, LB medium was solidified with 1.5 % agar. Overlay agar or agarose contained 0.7 %

agar or agarose respectively in LB medium.

Table 2.1 Bacterial strains and vectors

E.coli[a] strains	genotype or phenotype
DH5α	F⁻, *end*A⁻, *hsd*R⁻17(r^-_k,m^+_k), *sup*E44, *thi*$^{-1}$, λ⁻, *rec*A1, *gyr*A96, rel A1, Δ(*arg*F⁻*lac*zya)U169, ø80d*lac*ZΔM15
Y1090(r-)	Δ*lac*U169, *pro*A⁺, Δ*lon* *ara*D139, *str*A, *sup*F, [*trp*c22::Tn10], *hsd*R⁻, *hsd*M⁺, (pMC9, Amp^R,Tet^R), pMC9=pBR322-*lac*I⁺.]
LE392:	F⁻, *hsd*R514, *sup*E44, *sup*F58, *lac*Y1 or (*lac*IZY)6, *gal*K2, *gal*T22, *met*B1, *trp*R55
Vectors[a]	
pGEM-4	Amp^r
λgt11[b]	λ*lac*5 Δ*shn*dIIIλ2-3 *sr*Iλ3° c*Its*857 *sr*Iλ4° *nin*5 *sr*Iλ5° *S*am100

[a] from Promega, USA

[b] detailed map in Fig. 2-1

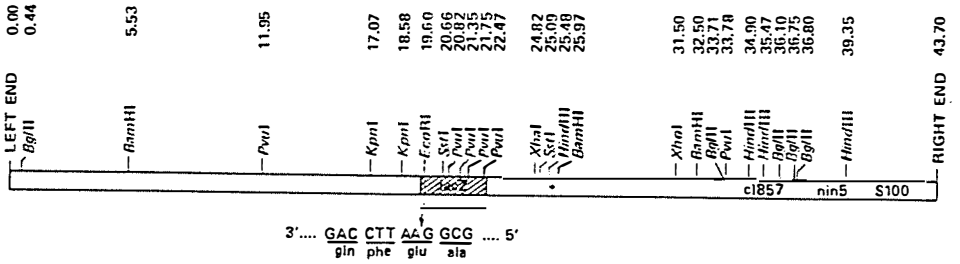


Fig. 2-1 Map of λgt11.

2.3 Biochemical methods

2.3.1 Isolation of lectins from tamarillo

2.3.1.1 Chitin affinity chromatography

Tamarillo fruits (400 g) (a local unnamed cultivar) were homogenized in 400 ml of buffer A (144 Mm NaH_2PO_4, 56 Mm Na_2HPO_4, 10 mM EDTA and 0.02% NaN_3; pH 7.2) in a Waring blender at low speed for 20 seconds, and centrifuged (9,000 rpm for 15 min). The supernatant was filtered through four layers of cheese cloth and ammonium sulphate was added to 65% saturation. The resulting suspension was stirred for 30 min and centrifuged at 9,000 *g* for 30 min at 4°C. The precipitate was dissolved in 40 ml of buffer B (13 mM citric acid, 13 mM Na_2HPO_4, 1 mM EDTA and 0,02% NaN_3; pH 3.6) and stirred for 30 min at room temperature, and centrifuged. The precipitate was discarded. The resulting solution was loaded onto a chitin column (1.8 x 14 cm) pre-equilibrated in the buffer B, which was washed with buffer B until the absorbance at 280 nm dropped to zero, A protein fraction was eluted with 5 ml of chitin hydrolysate solution (see below) at 30 ml/hr, The eluate was dialysed against buffer B and loaded on to a S-Sepharose (Pharmacia, Sweden) column (2.2 x 6.5 cm) equilibrated in the same buffer. The sample was eluted with a salt gradient (70 ml of buffer B - 70 ml of buffer B containing 1 M NaCl) at 15 ml/hr. Fractions (2 ml) of the peak corresponding to 0.42 M NaCl were combined and dialysed against water and freeze-dried. The other peak fractions, which were eluted at less than 0.42 M NaCl, were also combined and dialysed against water, and subsequently freeze-dried.

2.3.1.1.1 Preparation of chitin affinity column

Unbleached chitin (3 g) (US Biochemical Corporation, USA) was added to 100 ml 1 M NaOH solution and autoclaved for 15 min, and then washed extensively with water until the pH was neutral. The chitin was packed into a column (1.8 x 14 cm), and equilibrated with buffer B described above.

2.3.1.1.2 Preparation of chitin hydrolysate solution (chitin affinity column eluting solution)

Preparation was according to Rupley (1964): 40 g of chitin (USB, USA) which had been powdered in a mechanical grinder were stirred in 700 ml of ice cold concentrated HCl (11.6 M) for 2 hrs at 0°C, then the suspension was stirred for 2 hrs at 40°C and subsequently cooled to 0°C. The pH was brought to 1.0 with 50% NaOH, and the temperature was kept below 20°C. The filtrate of this suspension was applied to a charcoal-celite (1:1 by weight; 5 x 20 cm) column, which was then washed with water until the eluate was chloride free when tested with saturated silver nitrate, and was subsequently eluted with 65% ethanol. The eluate was dried in a rotary evaporator under reduced pressure. The dried hydrolysate was dissolved in 60 ml of PBS (0.1 M phosphate, 0.15 M NaCl and 0.02% NaN_3; pH 6.8). This hydrolysate preparation was used as the eluting solution for the chitin affinity column. It was analyzed by HPLC on a Waters Sugar-Pak 1 column (Waters, USA). The following carbohydrates were used as external standards: N-acetylglucosamine, N,N'-diacetylchitobiose, N,N',N"-tri-acetylchitotriose, N,N',N",N"'-tetraacetylchitotetraose. The chitin hydrolysate was found to contain: 60.1 mM N-acetyl-glucosamine, 4.2 mM N,N'-diacetylchitobiose, 18.8 mM N,N',N"-triacetylchitotriose and 5.6 mM N,N',N",N"'- tetraacetylchitotetraose.

2.3.1.2 Gel filtration chromatography

The ammonium sulphate precipitate described above was dissolved in buffer C (17 mM citric acid, 16 mM Na_2HPO_4, 50 mM NaCl and 0.02% NaN_3; pH 3.6), and loaded onto a Sephacryl 200 (Pharmacia, Sweden) gel filtration column (2.2 x 90 cm) at 15 ml/hr, which had been pre-equilibrated with buffer C. The fractions containing high agglutinating activity were combined, dialysed against water and freeze-dried. One of the dried samples was dissolved in 10 ml buffer D (36 mM Na_2HPO_4, 14 mM NaH_2PO_4 and 50 mM NaCl; pH 7.2) and loaded onto a Sephacryl 200 column (2.2 x 90 cm) which had been pre-equilibrated with buffer D. The resulting fractions containing high agglutinating activity were pooled and dialysed against water, then freeze-dried. All the procedures were done at room temperature except where otherwise indicated.

2.3.2 Haemagglutination and carbohydrate inhibition assay

These were performed in a microtitre plate. 25 µl of lectin sample was diluted serially (2 fold) in 25 µl PBS buffer [0.14 M NaCl, 0.01 M phosphate(K^+), pH 7.2] and mixed with 25 µl of 2% PBS washed rabbit erythrocytes. A haemagglutination unit was defined as the minimum amount of lectin in µg/ml giving positive reaction after 20 min at room temperature.

For carbohydrate inhibition assay, 10 µl of carbohydrate solution was diluted serially in 10 µl of PBS buffer, mixed with 10 µl of lectin solution containing 8 haemagglutination units. After the mixture was incubated for 30 minutes at room temperature, 20 µl of 2% PBS washed rabbit erythrocytes was added and mixed. The inhibitory activity was defined as the

minimum concentration of inhibitor required for complete inhibition of 8 haemagglutination units.

2.3.3 Mitogenic study

Mixed Micro-culture medium containing the following constituents for each blood sample was dispensed into a sterile screwcapped bottle: 4 ml of Eagle's medium containing penicillin and streptomycin, 1 ml of sterile human AB serum (heparinized), 0.1 ml of sterile M/I Hepes (pH adjusted to 7.0), 0.15 ml of phenol-free heparin, 0.1 ml of reconstituted lectin (10 mg/ml in sterile water). 0.4 ml of heparinized blood was then added. The cultures were incubated at 37°C for 72 hrs, shaken once a day by inversion. To each culture was added 0.15 ml of cholchicine solution (25 µg/ml) and the cultures were incubated at 37°C for 90 minutes and then centrifuged at 500 *g* for 5 min. Ten ml of potassium chloride solution (0.56 %, 37°C) was slowly added to resuspend the deposit while agitating constantly. The cultures were incubated at 37°C for 10 minutes, then centrifuged. Ten ml of freshly-prepared acetic alcohol (1 part glacial acetic acid; 3 parts methanol) was slowly added to the precipitate while agitating. The suspension was incubated at 4°C for 10 minutes before centrifugation. Another 5 ml of cold acetic alcohol was added to resuspend the precipitate and this washing step was repeated twice. Finally, 0.5 ml acetic alcohol was added to the precipitate. one or two drops of the re-suspended cell preparation were applied to the canter of a glass slide and allowed to spread and air dry. Cells were then stained with Giemsa or acetic acid-orcein and mounted in Euparal. The slides were examined under the microscope to identify the chromosomes.

2.3.4 Protein concentration assay

Protein concentration was determined according to Lowry (1951). $CuSO_4.5H_2O$ (0.5 g) and Na_3citrate (1 g) were dissolved in 100 ml water to make up reagent A; Na_2CO_3 (20 g) and NaOH (4 g) were dissolved water (1 l) to make up reagent B; Reagent B (50 ml) and reagent A (1 ml) to make up reagent C. Reagent D was made up by mixing 10 ml of Folin-Ciocalteau reagent with 10 ml water. Protein sample containing up to 0.5 mg of protein was added with 2.5 ml of reagent C, mixed and let stand for 5 min. Then, 0.25 ml of reagent D was added, mixed and let stand for 20-30 min. Absorbance (650 nm) was measured. Bovine serum albumin was used as standard.

The Bradford method (Bradford, 1976) using Coomassie Blue G250 was ineffective in the determination of protein concentration of the purified tamarillo lectin (CBL1).

2.3.5 Carbohydrate assay

Carbohydrate analysis was performed using the modified Dubois' method (Rao and Pattabiraman, 1989). 3.0 ml of sulphuric acid (sp.gr.1.84) was added to 1 ml of sugar or glycoprotein solution, and vortexed (the temperature reached rapidly a maximum of 115-118°C in 10-15 sec). After being cooled on ice for 2 min, the solution was mixed with 0.05 ml of 80% phenol, kept at 37°C for 30 min before its colour intensity (480 nm) was measured. Galactose was used as standard.

2.3.6 SDS-Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially according to Laemmli (1970). The following reagents for the separation gel were mixed and degassed for 5 min: 10 ml of 30 % acrylamide stock (300 g of acrylamide and 8 g of bisacrylamide were dissolved in water, final volume 1 litre), 9.8 ml water and 0.2 ml 10% ammonium persulphate (freshly prepared). After being degassed, the solution was mixed with 10 µl of TEMED by gently swirling, and poured into a glass sandwich (20 cm x 17 cm x 0.75 cm) until 3 cm from the top of the plate. 0.1% SDS solution was layered onto the surface of the gel solution. After the gel was polymerized, the SDS layer was removed by rinsing with water. A stacking gel solution was poured into the sandwich, which was prepared by mixing following reagents: 2.5 ml of 4 x upper buffer (0.5 M Tris HCl, pH 6.8, 0.04% SDS), 1.0 ml of the acrylamide stock, 6.4 ml of water, 0.2 ml of 10% ammonium persulphate, 10 µl of TEMED. A sample comb was inserted. After the stacking gel was polymerized, the comb was removed and the wells were rinsed with water. The gel sandwich was placed in a gel electrophoresis apparatus, the upper and lower buffer chambers were filled with 1 x running buffer (4 x running buffer: 60 g of Tris, 288 g of glycine, water up to 5 litres). Protein samples were prepared by boiling with equal volume of 2 x loading buffer for 5 min [0.5 ml of β-mercaptoethanol, 0.25 ml of 0.1% Bromophenol Blue (BpB), 4.0 ml 10% SDS, 5.3 ml of 2 x SB (12.5 ml of 4 x upper buffer, 20.0 ml glycerol, made up to 60 ml with water)]. 10-20 µl of sample per lane were loaded onto the well. Electrophoresis was carried out at 15 mA until the blue-dye front reached the bottom of the gel. After the glass plates were removed, the gel was stained with either Coomassie brilliant blue (R250) or silver ion. A set of protein molecular weight standards were used for molecular weight estimation.

Polyacrylamide gel electrophoresis was also performed using the same reagents in a Mini-Protein II Dual Slab Cell (Bio-Rad, USA) following the manufacture's instruction. The running conditions was 200 volts for 50 min at the room temperature.

2.3.6.1 Coomassie blue (R250) staining

The gel was stained for 30-60 min in 200 ml of staining solution (staining stock: 125 ml isopropanol, 50 ml acetic acid, 325 ml water, 1.25 g Coomassie brilliant blue R250). The gel was destained in several changes of a destaining solution (stock solution: 100 ml of methanol, 140 ml of acetic acid, water up to 2 litre). The process was carried out on a platform shaker.

2.3.6.2 Silver staining

Silver staining of protein gels was carried out according to Merril *et al.* (1981) using a silver staining kit (Bio-Rad, USA). The gel was fixed in 400 ml of fixing solution [40% methanol, 10% acetic acid(v/v)] for 30 min, and then transferred to 200 ml of another fixing solution (10% ethanol and 5% acetic acid) for 15 min. The fixation step was repeated once. After fixation, the gel was placed in 200 ml oxidizer for 5 min, and washed twice with deionized water for 5 min each. The gel was subsequently incubated with 200 ml of silver reagent for 20 min, rinsed with developer solution for 30 sec, placed in a 200 ml of freshly prepared developer solution for 5 min. The developing step could be extended by changing the developing solution until a reasonable intensity of protein bands had occurred. This developing process was stopped by placing the gel in 5% acetic acid. The whole

process was carried out on a platform shaker.

2.3.7 Isoelectric focusing (IEF)

Isoelectric focusing was performed using an LKB Multiphor 2117 gel electrophoresis apparatus (LKB, Sweden) [adapted from Righetti (1983)]. The following reagents were mixed: 0.825 ml of 30 % acrylamide stock solution (as described in Section 2.3.6); 4.0 ml of MilliQ water; 0.175 ml of carrier ampholytes ('Resolyte' pH 3.5-10) (BDH, England). After being degassed for 5 min, the solution was then mixed with 100 µl of 10 % ammonium persulphate and 13 µl of TEMED, and poured into a glass sandwich (11 cm x 8.5 cm x 0.75 cm). After the gel was polymerized, the upper glass plate of the gel sandwich was removed, and the gel with one glass plate at its bottom was placed on the cooling plate of the LKB Multiphor (4-0°C). After being connected with anode solution (1 M H_3PO_4) and cathode solution (1 M NaOH) using two pieces of 3 MM paper, the gel was pre-run at the following power supply settings: E = 1000 V; I = 17 mA; P = 12.5 W for 10 min. Then, 10 µl of each protein sample (0.5-1 mg/ml) and 0.5 µl of pI standards (Bio-Rad, USA) were applied onto the gel with sample application wicks (LKB, Sweden), and the electrophoresis was performed at the same conditions as described above for 90 min (the sample application pieces were removed after 30 min from the start of electrophoresis). After electrophoresis was completed, the gel was incubated with 200 ml of a fixing solution containing 11.5 % trichloroacetic acid and 3.45 % sulphosalicylic acid for one hr, and was stained with silver ion as described in Section 2.3.6.2.

2.3.8 Determination of protein size by gel filtration

CBL1 was run on a Sephadex G-75 column (2.2 cm x 90 cm) at a flow rate 15 ml/hr in buffer C and Buffer D as described in Section 2.3.1.2. The elution volume of CBL1 was compared to those of a set of protein molecular weight standards run under the same conditions.

2.3.9 Amino acid analysis

Amino acid analyses were carried out using a Beckman 119BL amino acid analyzer after hydrolysis of samples *in vacuo* in 6 M HCl for 24 hrs at 110 C. Cysteine contents were determined by analysis of cysteic acid after oxidation with performic acid (Hirs, 1967).

2.3.10 Tryptic digestion

CBL1 (11 mg) was dissolved in 1 ml of Tris buffer (0.2 M, pH 8.0) and mixed with 953 mg of guanadinium HCl to 6 M, and 15.4 mg DTT (100 mmoles). The reaction tube was flushed with N_2, sealed and kept in the dark by wrapping with aluminum foil. After being stirred for 2 hrs, the reaction was mixed with 52.5 mg of 4-vinyl pyridine, flushed with N_2, sealed, and kept in the dark. After stirred for another 2 hrs, the reaction was mixed with 1 ml of β-mercaptoethanol and incubated for 15 min. Then, the reaction was dialysed extensively against water (4 changes in 24 hrs) and freeze-dried.

The freeze-dried modified CBL1 was dissolved in 5 ml of 1 %

(w/v) NH_4HCO_4 and 0.05 mg of TPCK treated trypsin was added. Another 0.05 mg of TPCK treated trypsin was added after incubation at 37°C for 2 hrs, and incubation continued for another 2 hrs. The final digestion was freeze-dried.

2.3.11 HPLC separation of peptides

The tryptic digested CBL1 was dissolved in 1 ml of solution A [0.01% trifluoroacetic acid (TFA) in water], then the solution was filtered through a 0.2 μm nylon filter. 100 μl samples were loaded onto a HPLC (Spectra-Physics) fitted with a reverse phase column (vydak, C18). The separation condition was as follows: flow rate 1 ml/min; elution gradient: 100% solution A at 0 min, 50% solution A, 50% solution B (0.08% TFA in acetonitrile) at 50 min. The samples under each peak was collected in a plastic tube and freeze-dried.

2.3.12 Gas-phase protein sequencing

Gas-Phase sequencing was performed using an Applied Biosystems 470A protein sequencer connected to an Applied Biosystems 120A PTH analyzer. Standard protocols from the manufacturer were used throughout.

2.4 Immunological methods

2.4.1 Antiserum preparation

CBL1 (0.5 mg/ml, 0.5 ml) was mixed with equal volume of Freund's complete adjuvant (Difco), and injected

subcutaneously into a New Zealand White Rabbit. Six weeks after the first injection, 0.5 ml of CBL1 was mixed with an equal volume of Freund's incomplete adjuvant and injected subcutaneously into the New Zealand white rabbit. Bleeding was done intracardially after three weeks. Fifty ml of rabbit blood was incubated at 37°C for 3-4 hrs to induce cell clotting, with occasional stirring to break up the coagulation, and then left at 4°C overnight. The blood sample was centrifuged at 1000 *g* for 5 min, and the supernatant was dispensed into small aliquots and stored at -70 °C before use.

2.4.2 Double immunological diffusion test (Ouchterlony test)

Twenty mg of agarose (Electrophoresis Grade, BRL, USA) was added to 2 ml of PBS buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.15 M NaCl; pH 6.8) or 2 ml of chitin hydrolysate solution (Section 2.1.1.4). The suspension was heated at 100°C for 2 min before pouring onto horizontal microscopic slides (2.5 cm x 7.5 cm). After the gel had solidified, six peripheral wells and a canter well were made using a template. The distance between the adjacent wells was 0.4 cm. Ten µl of undiluted antiserum was placed in the canter well and 10 µl (5-10 µg) of lectin sample was placed in these peripheral wells. Slides was incubated in a moisture chamber at 4°C overnight to 4 days. The precipitin lines formed among between these wells were photographically recorded.

2.4.3 Western blotting

The western blotting method was performed using a mini trans-blot electrophoretic transfer cell (Bio-Rad, USA) (Towbin *et al.*, 1979; Burnett, 1981). Standard protocols from

the manufacturer were used throughout. After equilibration in the transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for 15 min, the following components were assembled into a blotting sandwich in the transfer buffer in the order from the bottom: the grey panel of the cassette, fibre pad, 2 pieces of 3MM paper, SDS-PAGE gel, nitrocellulose membrane, 2 pieces of 3MM paper, fibre pad, the clear panel of the cassette. After being locked, the cassette was transferred to the buffer chamber filled up with the transfer buffer and installed with a Bio-ice cooling unit. The protein transfer was achieved in an hour at 100 volts, 150 mA. Indirect immunological detection of antigens on the membrane was performed according to the method for immunological screening for cDNA library. Total proteins on the membrane were detected using a colloidal gold staining kit (Rohringer and Holden, 1985): the nitrocellulose membrane was washed with two changes of 100 ml of TTBS solution (20 mM Tris, 500 mM NaCl, 0.3% Tween-20; pH 7.5) for 20 min each. The membrane was then washed with 3 changes of 100 ml deionized water for 10 min each. The membrane was then placed on a horizontal glass plate and colloidal gold reagent was applied. The membrane was rinsed with water before reasonable intensity of protein bands was reached.

2.4.4 Immunocytochemical localization

Young tamarillo fruit (2 cm x 3 cm) were hand-sectioned using a razor blade. The sections were mounted on 10% PVA glue coated slides, fixed in methanol for 5 min, and air-dried. The sections were then incubated in PBSB [0.1 M phosphate buffered saline (pH 7.5) supplemented with 1% bovine serum albumin] for 5 min. The PBS solution was then removed and the section was incubated for 1.5 hrs in anti-CBL1 serum which was diluted 20 times in PBSB. The sections were subsequently washed with three changes of PBS for 1 min each. After the

wash, the sections were incubated for 2 hrs in biotinylated donkey anti-rabbit IgG which was diluted 200 times in PBSB. After repeated washing in PBS as described above, the sections were incubated in the dark for 40 min in streptavidin-biotinylated phycoerythrin solution which was diluted 50 times in PBSB, followed by PBS washes as described, then incubated with stabilizer for 20 min in dim light. Excess stabilizer was drained and wiped off around the sample. The slides were completely dried in a dark 37°C chamber for 10-20 min. The sections were covered with coverslips and viewed using a Zeiss Axiophot fluorescence microscope (Germany) with the following filter combinations: Excitation 540-552 nm; dichroic mirror, 580 nm; Barrier, 590 nm.

Specificity of labelling was assessed with 6 controls: 1, incubation of sections with PBS containing BSA but without the antiserum, secondary antibody and streptavidin-biotinylated phycoerythrin complex steps; 2, incubation of sections with preimmune or nonimmune serum with the secondary antibody and streptavidin-biotinylated phycoerythrin complex steps; 3, incubation of sections with the antiserum absorbed with excessive CBL1, secondary antibody and streptavidin-biotinylated phycoerythrin complex steps; 4, incubation of sections without the antiserum step but with the secondary antibody and streptavidin-biotinylated phycoerythrin steps; 5, incubation of sections with the antiserum, followed by 1 % BSA in PBS and the streptavidin-biotinylated phycoerythrin complex steps (i.e. without secondary antibody step); 6, incubation of sections without the antiserum and secondary antibody steps, but with the streptavidin-biotinylated phycoerythrin complex step.

2.5 Molecular biological methods

2.5.1 Poly(A^+) RNA preparation

2.5.1.1 Preparation of the frozen fruits

Young fruits about a month old were washed in water and 70% ethanol and cut into small pieces (about 1 cm^3), then frozen immediately in liquid air or liquid nitrogen and stored at -70°C.

2.5.1.2 Total RNA isolation

The method used was mainly according to De Vries *et al.* (1986). All the glassware used for the purification of RNA was baked at 160°C for 24 hrs. The polypropylene centrifuge tube was filled with deionized water containing 0.2 % diethylpyrocarbonate (DEPC), vigorously shaken and autoclaved. Buffer, water and any other reagents were also treated with DEPC before autoclaved. Ten g of young fruits from storage at -70°C was ground in liquid air in a pre-cooled mortar and pestle until a fine homogenous powder was obtained, which was then transferred to a 100 ml beaker, mixed with 20 ml of phenol/extraction buffer [1:1 mixture of RNA extraction buffer (100 mM LiCl, 1% SDS, 100 mM Tris-NaOH, 10 mM EDTA, pH 9.0) and distilled phenol containing 0.1% hydroxyquinoline]. The suspension was stirred with a magnetic stirrer at 300 rpm/min for 5 min, then 10 ml of chloroform was added and stirred continuously for another 30 min before centrifugation at 20,000 g for 30 min at 25 °C. The aqueous upper phase was recovered, mixed with 10 ml of chloroform, shaken, and centrifuged as described above. The aqueous

phase was recovered, mixed with 1/3 volume of 8 M LiCl, and incubated at 4 °C for 16-48 hrs. RNA was pelleted by centrifugation at 12,000 *g* for 30 min at 4°C, washed once with 2 M LiCl at 4°C and twice with 80% ethanol, and stored at -20°C.

2.5.1.3 Poly(A^+) RNA isolation

This method was mainly according to Cox and Goldberg (1988). Oligo-dT cellulose (Pharmacia, Sweden) (50 mg) were packed in a siliconized column (0.7 cm x 10 cm) , washed twice with 10 ml of the following solutions: 0.05% DEPC; 0.1 M NaOH; elution buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1% SDS, pH 7.6; treated with DEPC and autoclaved); the binding buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.1% SDS, pH 7.6; treated with DEPC and autoclaved). RNA was resuspended in elution buffer on ice to give a final concentration less than 0.5 mg/ml, which was then heated at 68°C for 3 min, cooled quickly on ice for at least one min, brought to room temperature and added with NaCl to a final concentration of 0.5 M before loaded onto the column (1 ml/min), The fractions were collected in siliconized tubes on ice. These fractions were combined and then loaded onto the affinity column again. The column was washed with binding buffer until all unbound RNA was removed, which was monitored by comparing the A_{260} of the flow with that of binding buffer. The poly(A^+) RNAs were eluted with the elution buffer, collected as aliquots on ice in siliconized tubes. The column was regenerated by washing in the solutions described above. The poly(A^+) RNA sample was heated at 68°C for 3 min, and subjected to another round of oligo(dT) chromatography as described above. The final poly(A^+) RNA was added with 1/3 volume of sodium acetate and 2,5 volume of ethanol, mixed and placed in -20°C overnight. RNA was collected by centrifuging at 12,000 *g* for 15 min at 4°C, which was then washed with 70% ethanol, dissolved in

DEPC treated sterilized water at 1 µg/µl and Stored at -70°C.

2.5.2 *In vitro* translation of mRNA

In vitro translation of mRNA was performed using a wheat germ translation system (Promega, USA). The following reagents were mixed in a sterile tube: 25 µl of wheat germ extract, 4 µl of 1 M amino acid mixture (minus methionine), 1 µl of poly(A^+) RNA, 1 µl of [^{32}S]-methionine (1250 Ci/mmole), 13.5 µl of nuclease free water, 5 µl of potassium acetate (1 M). The reaction was incubated at room temperature for 2 hrs. The translation products were run on a SDS-PAGE gel, which was then soaked for 3 hrs in 500 ml of a solution containing 3 % glycerol (w/v), 40 % methanol, 10 % acetic acid before drying in a gel drier. The gel was analyzed by autoradiography. Rabbit globin mRNA was used as positive control.

2.5.3 RNA quantisation

If A_{260}/A_{280} for RNA solution was approximately at 2.0, the quantity of RNA was calculated by measuring the A_{260} of RNA solution (1 unit of A_{260} = 40 µg/ml). If A_{260}/A_{280} for the RNA solution was less than 2.0, the RNA was subjected to further purification by phenol/chloroform extraction.

2.5.4 Siliconization of glassware and plastic materials

This method was according to Seed (1988). Materials were placed in a desiccator into which a tube containing dimethyldichlorosilane (BDH, UK) was placed. The desiccator was connected to vacuum suction until the siliconizing

reagent reached boiling (about 5 min), the vacuum was then released as quickly as possible before being re-connected to vacuum suction until the reagent reached boiling again. The desiccator was left under vacuum for 2 hrs to overnight. The siliconized materials were autoclaved before use.

2.5.5 cDNA library construction

Synthesis and cloning of the cDNA of tamarillo fruit was performed according to Okayama and Berg (1982), Gubler and Hoffman (1983) using a cDNA synthesis kit (RiboClone) from Promega Corporation. The procedures used were mainly based on the manufacturer's instruction, a diagram is shown in Fig. 2-2.

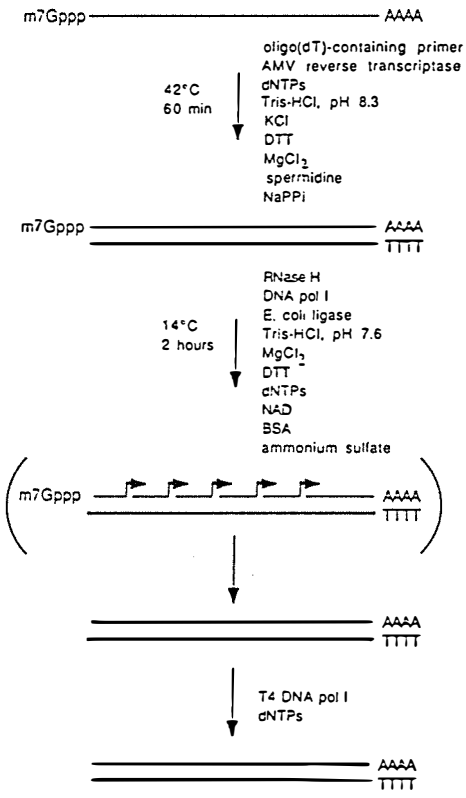


Fig. 2-2 A diagram of cDNA synthesis.

2.5.5.1 cDNA synthesis

A 1:10 dilution of AMV reverse transcriptase was prepared using the dilution buffer (10 µl) and stored on ice for 30 min. In a sterile RNase free microcentrifuge tube, 0.6 µl of primer (0.5 µg/ml) and 1 µl of poly(A^+) RNA (1 µg/µl) from tamarillo fruits were mixed and heated to 75°C for 5 min before cooling slowly to room temperature. The following components were added to the annealed primer/template in the order shown to a total volume of 25 µl: 4.5 µl of nuclease free water, 2.5µl of 10 x first strand buffer, 2.5 µl of 100 mM DTT, 2.5 µl of dNTP mixture, 0.5 µl of RNasin ribonuclease inhibitor, 2.5 µl of 40 mM Na pyrophosphate, 10µl reverse transcriptase (22 units). The reaction was mixed gently. 5 µl of sample were removed to another tube containing 2-5 µCi of [α^{32}-P]dCTP (0.5 µl), which was used for calculating the yield of the first strand reaction. Both tubes were incubated at 42°C for 60 min, and then placed on ice. To the incorporation reaction tube, 1µl of 0.2 M EDTA and water was added to total volume of 100µl, and stored on ice.

The following components were added to the first strand reaction tube for the second strand synthesis to a total volume of 100 µl: 52.7 µl of nuclease free water, 10 µl of 10 x second strand buffer, 3 µl of 100 mM DTT, 10 µl of 1 mM NAD, 0.5 µl of [α^{32}-]dCTP (2-5 µCi*), 2.3 µl of *E. coli* DNA polymerase I (23 units), 1 µl of *E. coli* ligase (1 unit), 0.5 µl of *E. coli* RNase H (0.8 units). The reaction was incubated at 14 °C for three hours followed by heat at 70°C for 10 min, and placed on ice. Five µl of the reaction was removed to another tube for incorporation assays. Four units of T_4 DNA polymerase was added to the remaining reaction, which was then incubated at 37°C for 10 min, stopped by adding 10 µl of 0.2 M EDTA and placed on ice. The reaction which was then mixed with an equal volume of phenol: Chloroform:isoamyl

alcohol [25:24:1 (v/v/v)], and centrifuged for 1 min at room temperature. The aqueous phase was transferred to a fresh tube, and mixed with 0.5 volumes of 7.5 M ammonium acetate and three volumes of cold ethanol (-20°C), incubated on ice for 15 min. DNA precipitate was collected by centrifugation for 15 min at 4°C, and washed with 70 % cold ethanol, dried under vacuum and dissolved in 10 µl deionized water or TE buffer. 0.5 µl of DNA were removed to another tube for alkaline gel analysis.

2.3.5.2 Incorporation assays, calculations and gel analysis

One µl of the diluted first strand tracer reaction (100 µl in total) and 1 µl of the second strand tracer reaction were placed on glass fibre filters and air-dried. Two µl of each reactions were added to tubes containing 10 µl of 1 mg/ml solution of herring sperm, mixed with 0.5 ml of 5 % trichloroacetic acid (TCA), and incubated on ice for 5-30 min. These samples were filtered through glass filters, which were subsequently washed 3 times with 5 ml cold 5 % TCA, rinsed with acetone or ethanol, and air-dried. The filters were then placed in 10 ml of the scintillation fluid, and radioactive counts were recorded.

The formula for calculating the yield is as follows:

$$\text{first and second strand yield (\%)} = \frac{\text{incorporated cpm} \times 10}{\text{total cpm} \times 20}$$

$$\text{\% conversion to double stranded cDNA} = \frac{\text{ng second cDNA synthesized}}{\text{ng first strand cDNA synthesized}}$$

2.5.5.3 Alkaline gel electrophoresis

This method was according to Maniatis *et al.*(1982). 97 µl of the first strand trace reaction (a total volume of 100 µl) were extracted with phenol/chloroform and precipitated as described above. The first strand DNA was finally dissolved in 10 µl water. 5 µl of the first strand DNA and 0.5 µl of the ds DNA (a total volume of 10 µl) were mixed with equal volume of 2 X sample buffer (20 mM NaOH, 20 % glycerol, 0.025 % freshly added bromophenol blue), and loaded on a 1 % alkaline agarose gel [1 % agarose was dissolved in 50 mM NaCl, 1 mM EDTA, poured into a gel, which was equilibrated for at least 30 min in running buffer (30 mM NaOH, 1 mM EDTA) before use]. Electrophoresis was proceeded at 7.5 V/cm until the dye reached 2/3 of the way across the gel. The gel was then incubated in 200 ml of 7 % trichloroacetic acid (TCA) for 30 min before being dried in a dryer. The size distribution was analyzed by autoradiography at -70 °C with an intensifying screen.

2.5.5.4 Methylation of cDNAs

cDNA solution (6 µl) was placed in a tube, and mixed with the following reagents: 1 µl of 1 mM S-adensosyl-methionine [made by diluting stock solution (10 mM) with water], 1 µl of 10 x *Eco*R1 methylase buffer, 1 µl of BSA (1 mg/ml), 1 µl of *Eco*R1 methylase. The reaction was incubated at 37 °C for 15 min, stopped by incubation at 70 °C for 10 min, then vortexed with 1 volume of phenol: chloroform (1:1 part buffer saturated, pH 7-8, 1 part chloroform /isoamylalcohol, 24:1), and centrifuged at 12,000 *g* for 3 min. The aqueous phase was recovered to a fresh tube and extracted with phenol/ chloroform as described. The aqueous phase was placed in a tube and mixed with 1/10 volume 3 M NaOAc (pH 5.2), 2 volume

of 100% alcohol, and incubated at -70°C for 30 min. DNA precipitate was collected by centrifugation at 12,000 *g* for 15 min, and washed with 70% ethanol, dried under vacuum, and resuspend in 4 µl of TE buffer (pH 8.0) or deionized water. The final concentration of the double stranded cDNA was approximately 100 ng/µl.

2.5.5.5 Linker ligation

The following components were mixed in a tube: 1 µl of ligation buffer, 1 µl of BSA (1 mg/ml), 2 µl DNA (100 ng/µl), 1 µl of diluted phosphorylated *Eco*R1 linker (1/10 of stock), 2.5 Weiss units (1.3 µl of the stock) of T_4 DNA ligase, and 3.7 µl of water, The reaction was incubated at 15°C for 6-18 hours and stopped by incubation at 70°C for 10 min.

2.5.5.6 Digestion with *Eco*R1

After inactivation, the ligation reaction was cooled on ice and mixed with the following reagents: 3 µl of *Eco*R1 buffer (x 10), 17 µl of water and 10 units of *Eco*R1 (0.5 µl of stock). The digestion was proceeded at 37 °C for 1.5 hrs and stopped by mixing with 3 µl of 0.2 M EDTA. DNA sample was extracted with phenol/chloroform as described.

2.5.5.7 Removal of unligated linkers

The unligated linkers were removed by a spin column procedure: restriction digestion reaction was applied (Section 2.3.4.4) to the top of the gel bed of spin column[1], which was placed in a tube and centrifuged in a horizontal

rotor at 800 *g* for 5 min. The eluate was mixed with 0.5 volume of 7.5 M ammonium acetate, 2 volumes of ethanol, and incubated at -20 °C for 30 min. DNA precipitate was collected as described and resuspended in 3 µl of water for further reaction.

[1] Preparation of spin column: Sephacryl S-400 slurry (suspended in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) was applied to a vertical column (provided by Promega), and the buffer was allowed to drain. The final height of the gel bed should reach the neck of the column at the "ring" mark (about 1.2 ml of 1:1 slurry). The column tip was then placed inside a tube, and the assembly was centrifuged in a swinging bucket rotor at 800 *g* for 5 min. The column was ready for use.

2.5.5.8 Ligation of cDNAs to λgt11 arms and *in vitro* package of ligated λgt11 viruses

The following reagents were mixed (total volume of 5 µl): 1 µl of λgt11 arms (0.5 µg), 3 µl of *Eco*R1 digested cDNAs, 0.5 µl of ligation buffer (x 10), and 0.5 µl of T_4 DNA ligase (1 Weiss unit). After incubated at 15°C for 3 hrs or overnight. The entire ligation reaction was mixed with 25 µl of packagene extract and incubated at 22°C for 2 hours, followed by addition of 0.5 ml of phage buffer (20 mM Tris, 100 mM NaCl, 10 mM $MgSO_4$, pH 7.5) and 25 µl of chloroform. The packaged phage solution was kept at 4°C for up to 3 weeks.

Appropriate dilutions of the packaged solution were made in phage buffer. 100 µl of the diluted phage was mixed with 100 µl of Y1090 bacteria and incubated at 37°C for 20 min. Then the suspension was mixed with 3 ml of molten H top agarose (45 °C) supplemented with 40 µl of X-Gal stock solution (20 mg/ml in dimethylformamide and 5 µl of IPTG stock solution (200 mg/ml in water), poured onto a LB plate. After the top agar was hardened, the plate was incubated at 42°C for 4 hours and then at 37°C for another 4 hours. The number of

white and blue plaques was counted and the titre of the phages and percentage of the recombinant phages were calculated.

2.5.6 Amplification of cDNA library

The method was according to Klickstein (1987). A single colony of LE392 was inoculated in 5 ml of LB medium supplemented with 0.2% maltose and 10mM $MgSO_4$, grown over night at 37 °C while shaking. The bacteria cells were collected by centrifugation and resuspended in sterile 0.01 M $MgSO_4$ to an appropriate density (OD600 was 2), and stored at 4°C for up to 3 weeks. 100 µl of unamplified library was mixed with 2 ml of LE392 cells, incubated for 20 min at 37°C. Each aliquot (200 µl) was mixed with 3 ml of soft agar or agarose, and immediately poured into a LB plate. After incubated at 37°C for 10-12 hrs, each plate was applied with 10 ml of SM buffer (phage buffer supplemented with 0.1 % gelatin), and shaken gently for 4 hrs. The phage eluate from each plate was then combined into a single 500 ml polypropylene tube, and centrifuged at 5,000 rpm for 5 min. The phage solution (amplified cDNA library) was then stored at 4°C in a glass bottle containing 0.5 ml of chloroform. 930 µl of amplified library (without chloroform) was mixed with 70 µl of dimethyl sulphoxide (DMSO) in a plastic centrifuge tube and stored at -70°C.

2.5.7 Immunological screening of cDNA library

2.5.7.1 Screening

This method was according to Sambrook *et al.* (1989). Each 100 µl of Y1090 overnight culture was mixed with 100 µl of phage solution containing 1 x 10^4 plaque forming units (pfu) of the λgt 11 library, incubated at 37°C for 20 minutes, then mixed with 2.5 ml of molten top agarose and poured onto a LB plate. The plates were allowed to set and incubated for 3.5 hours at 42°C. After being incubated for 3.5 hrs at 42°C, the plates were removed to room temperature, and quickly overlayed with the IPTG-impregnated nitrocellulose filters [which were numbered and soaked in 10 mM isopropylthio-β-D-galactoside (IPTG) for a few minutes, dried on a pad of adsorption paper], and further incubated in 37°C incubator for another 4-5 hrs. At the end of incubation, the plates were transferred to room temperature. Each filter was marked in at least three asymmetric locations, peeled off the plates, immersed in large volume of a blocking buffer [TNT buffer (10 mM Tris Cl, 150 mM NaCl, 0.05% Tween 20, pH 8.0) containing 3 % bovine serum albumin (A7030, Sigma, USA) and 0.05% NaN_3], and gently shaken for 30 min. At the end of the incubation, the filters were transferred to petri dishes (two filters per petri dish) containing 10 ml of the diluted anti-CBL1 serum (1:1000 in the blocking buffer), gently shaken at 4°C overnight. The filters were washed in 10 ml of the following buffers below in the given order for 10 min each: TNT + 0.1% bovine serum albumin, TNT + 0.1% bovine serum albumin + 0.1% Nonidet P-40, TNT + 0.1% bovine serum albumin. These filters were then incubated with 10 ml of diluted goat anti-rabbit IgG (human-adsorbed) conjugated with alkaline phosphatase (1:3300 in the blocking buffer), gently shaken at 4°C cold room for 1-2 hrs, and then washed as described. After the last wash, the filters were blotted on paper towels and

incubated for 1 hr in BCIP/NBT developing solution containing 33 µl of the NBT stock (50 mg/ml in 70% dimethylformamide), 16.5 µl of BCIP stock (50 mg/ml in 100% dimethylformamide) and 10 ml of alkaline phosphatase buffer (100 mM Tris Cl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$)]. Ring shaped spots with intense purple colour might occurring at some sites of plaques were regarded as positive ones.

2.5.7.2 Identification and purification of positive plaques

A plug of agar were removed from positive plaque site which was located by comparing the plaque positions in the filter with those in the plate. It was transferred to 1 ml of SM containing 2 drops of chloroform and incubated for several hours at 4°C. Bacteriophages in the elute replated so as to obtain approximately 3000 plaques and screened for positive plaques. Screening and plating were repeated until a homogeneous population of immuno-positive recombinant bacteriophages was obtained.

2.5.8 Lambda phage preparation

Lambda phage lysate preparation was according to Maniatis *et al.*(1982). About 10^5 bacteriophages were mixed with 0.1 ml of plating bacteria, incubated for 20 min. The mixture was plated as described. The plate was incubated at 37 °C for 10-12 hrs to achieve confluent lysis, then added with 2 ml of SM buffer and shaken for 1 hr at 4°C. The SM buffer was finally transferred to a centrifuge tube, mixed with 0.1 ml of chloroform, and then shaken for 15 min before being centrifuged at 4,000 *g* for 10 min at 4°C. The supernatant was recovered and used for phage DNA preparation.

Phage preparation was according to Pohl (1984). Ten g DEAE-cellulose was suspended in about 200 ml of 0.05 N HCl, which was neutralized with about 10 N NaOH. After washing 3-4 times with a five-fold volume of saline, DEAE cellulose was resuspended in saline [75% (v/v) DEAE cellulose and 25% (v/v) saline]. 0.6 ml of the DEAE cellulose suspension was mixed with 0.6 ml of phage lysate (titre 5 x 10^3 pfu/ml) in a tube, centrifuged briefly. The supernatant was recovered, mixed with 1 µg/ml RNAase, and incubated for 30 min at 37°C. At the end of the incubation, 120 µl of a protein denaturing buffer [10 mM Tris-HCl pH 8, 2.5 % SDS and 0.25 M EDTA] was then added, incubated at 70°C for 15 minutes, allowed to cool at room temperature for 10 minutes. Proteins were aggregated by vortexing with 75 µl of 5 M potassium acetate and removed by centrifugation after the suspension incubated on ice for 15 minutes. The supernatant (1.0 ml) was recovered to another tube, mixed with 0.625 ml of isopropanol. DNA was precipitated as described, and resuspended in 10-50 µl of water.

2.5.9 Restriction enzyme digestion

Enzyme digestion was carried out with type II restriction endonucleases. One volume of 10 x restriction enzyme buffer was mixed with 7 volume of DNA solution (1 µg) in water. One volume of 1 mg/ml BSA and 1 volume of restriction enzyme (1 unit) was added into the mixture and incubated at 37°C for 1 hr. One µl of the volume was loaded into a mini agarose gel to check for reaction completion. If the digestion was completed, the reaction mixture was heated at 65°C for 15 min for inactivating the enzyme. *Eco*RI, *Mlu*I, *Sac*I, *Hinc*II, *Sph*I, *Pst*I, *Xba*I, *Bam*HI and *Hind*III were used according to the instruction of the supplier.

2.5.10 Plasmid isolation methods

2.5.10.1 Alkaline lysis method

This method was according to Sambrook *et al.* (1989). Three ml of overnight culture was centrifuged for 5 min to pellet the cells, which was resuspended by vigorous vortexing in 100 µl of ice cold Solution I (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0), followed by mixing with Solution II (2 N NaOH, 1 % SDS) and stored on ice. Ice-cold Solution III (150 µl) (5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml water) was added and gently mixed the alkaline suspension. After incubation on ice for 3-5 min, the tube was then centrifuged at 12,000 *g* for 5 min. The supernatant was recovered and mixed with 2 volumes of 100 % ethanol. DNA was precipitated as described, and dissolved in TE buffer or water.

2.5.10.2 Circleprep method

Circleprep method was performed using a Circleprep kit (Bio 101, USA) according to the manufacturer's instruction. Bacteria lysis was basically the same as alkaline lysis. The DNA pellet (from 50 ml starting culture) was dissolved in 0.5 ml water, incubated in a boiling water bath for 3-5 min, subsequently cooled in an ice-water bath for 2 min, followed by mixing with 300 µl of LiCl (provided by the manufacturer). The suspension was incubated at room temperature for 5 min and centrifuged for 2 min to pellet rRNA and ssDNA. The supernatant was recovered, mixed with 700 µl of isopropanol, and centrifuged. The pellet was dissolved in 0.5 ml of water, mixed with 300 µl of LiCl and 75 µl of the homogeneous suspension of Circleprep glassmilk. The milky suspension was

incubated for 5 min (occasionally shaken), subsequently centrifuged to collect DNA/glassmilk complex. The pellet was resuspended in the binding buffer (provided by the manufacturer) and precipitated by brief centrifugation, this wash step was repeated twice. Then, the DNA/glassmilk complex was resuspended in the washing buffer (provided by the manufacturer) and precipitated by brief centrifugation. This step was repeated twice. After drying under vacuum, the DNA/glassmilk complex was resuspended in 100 µl of water, and incubated at 55 °C for 5 min. DNA supernatant was recovered by brief centrifugation. The DNA sample prepared by this method was used for its *Exo*III deletion and sequencing.

2.5.10.3 Equilibrium centrifugation in CsCl-ethidium bromide gradient

This method was according to Sambrook *et al* (1989). Overnight culture (150 ml) in LB medium supplemented with ampicillin (60 µg/ml) was centrifuged at 9000 rpm for 5 min at 4°C to collect bacteria cells, which was resuspended in 20 ml STE buffer [0.1 M NaCl, 10 mM Tris.Cl (pH 8.0), 1 mM EDTA (pH 8.0)], and centrifuged as described. The cells were then resuspended in 18 ml of solution I [50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0)], then mixed with 2 ml of a freshly prepared solution of lysozyme [10 mg/ml in Tris Cl (pH8.0)] and 40 ml of freshly prepared Solution II (0.2 N NaOH, 1% SDS). The cell suspension was shaken several times by inversion, and incubated at room temperature for 5-10 min before being mixed with 20 ml of ice cold Solution III [5 potassium acetate 60 ml; glacial acetic acid 11.5 ml; water 28.5 ml]. After storage on ice for 5-10 min, the bacterial lysate was centrifuged at 9000 rpm for 10 min at 4 °C. The supernatant was recovered, mixed with 0.6 volume of isopropanol or 2 volume of ethanol, and incubated for 10 min. DNA was precipitated as described and dissolved in 5 ml TE

(pH 8.0).

Solid CsCl (5.25 g) was dissolved in 5 ml of DNA sample. 5 ml of CsCl/DNA was mixed with 375 µl of ethidium bromide (10 mg/ml in water), and centrifuged at 12,000 rpm for 15 min at room temperature. The supernatant was recovered (its refractive index was between 1.3860-1.393) and loaded in the ultracentrifuge tube. The tubes were sealed, balanced and loaded into a Sorvall TV865 Rotor. Ultracentrifugation was performed in a Sorvall ultracentrifuge at 60,000 rpm at 14°C for 5 hrs. Closed circular plasmid DNA was drawn out from the centrifuge tube using a syringe under long wavelength uv light. The DNA solution was extracted with an equal volume of isoamyl alcohol until the pink colour was disappeared in the aqueous phase. Finally, the DNA solution was dialysed extensively against water for 48 hrs before use. The closed circular plasmid DNA sample prepared by this method was used for sequencing.

2.5.11 DNA subcloning and plasmid transformation

2.5.11.1 DNA subcloning

DNA of interest was digested with *Eco*RI. After completion, the reaction was incubated at 70 °C for 15 min. DNA was extracted with phenol/chloroform, precipitated as described, and dissolved in appropriate volume of water. About equal molar digested DNA of interest and digested pGEM-4Z DNA were mixed with 1/10 volume of 10 x ligase buffer and 3 units of ligase, incubated at 15 °C overnight. The ligated DNA was used for transformation.

2.5.11.2 Plasmid transformation

Ligation mixture (10 µl) was mixed with 50 µl of competent cells, incubated on ice for 30 min, heat-shocked for 2 min at 42°C, and placed in ice for 2 min. The suspension was transferred to room temperature and mixed with 0.5 ml of LB medium. After shaking at 37°C for 1 hr, the entire culture or a diluted portion was then mixed with 2 ml LB molten soft agar in 47°C [soft agar might be supplemented with 10 µl of X-gal stock for screening recombinant plasmid], which was immediately poured onto a LB plate supplemented with ampicillin at 100 µg/ml. The plate was incubated in 37°C for 12-18 hrs. White colonies were picked up, their plasmid DNAs were extracted and digested with *Eco*R1. DNA was analyzed in a mini agarose gel.

2.5.12 DNA agarose electrophoresis

Four volume of DNA and 1 volume of gel loading dye (0.25 % bromophenol blue, 40 % sucrose in water) were mixed and analyzed by electrophoresis in 0.8 % to 2 % mini agarose gels using TBE buffer [89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH 8.0]. The gels were stained with 0.5 µg/ml ethidium bromide, and DNA was detected with a short wavelength UV transilluminator.

2.5.13 *Exo*III deletion of cDNA

*Exo*III deletion of DNA was performed according to Henikoff (1984) using Erase-a-Base system (Promega, USA). A diagram is shown in Fig. 2-3. A 0.025 volume of 2 M sodium acetate (pH 4.0) was mixed with a recombinant pGEM-4Z with cDNA inserted

at its *Eco*RI site (5 µg, prepared by Circle prep method), and NaCl to a final concentration of 75 mM. The DNA was mixed with an equal volume of redistilled phenol equilibrated with 50 mM sodium acetate (pH 4.0), vortexed for 1 min and allowed to sit for 1 min, vortexed again for 1 min, and centrifuged at 10,000 *g* for 5 min. The aqueous phase was recovered. This phenol extraction step was repeated for two times to remove most of the nicked or linear plasmids. The volume of aqueous phase was adjusted by adding deionized water to maintain the constant volume of initial DNA solution. When the extraction was completed, the DNA supernatant was mixed with 0.05 volume of 1M Tris (pH 8,6). DNA was precipitated as described, and dissolved in deionized water. Eight units of each of the restriction enzymes *Sph*I and *Xba*I were used for double digestion of the recombinant pGEM-4Z (2 µg). The double digestion was done at the same time using the reaction buffer for *Xba*I. Digested DNA was precipitated as described, dissolved in 7.3 µl of water, then mixed with 0.7 µl of 10 x *Exo*III buffer. Meanwhile, 4 tubes were prepared with 7.5 µl of S 1 mix (for 5 time points: 34.4 µl of deionized water, 5.4 µl of 7.4 x S 1 buffer, 12 units of S1 nuclease) and stored on ice. The DNA was then warmed to 25°C in a water bath, mixed with 360 units of *Exo*III. Samples (2.5 µl) at 2 min intervals were taken and pipetted into the S1 tubes on ice. The samples were quickly mixed by pipetting up and down. After all 4 samples were taken, the S1 tubes were transferred to room temperature, incubated for 30 min, then mixed with 1 µl of stop buffer and heated at 70°C for 10 min. Two µl of samples from each time point were removed for analysis on a 0.8% agarose gel to determine the extent of *Exo*III deletion. Meanwhile, the remaining samples were incubated at 37°C, and each of them was mixed with 1 µl of Klenow mix [30 µl of 1 x klenow buffer, 3-5 units of klenow DNA polymerase], and incubated for 3 min. Each tube was then added with 1 µl of dNTP mix (Promega, 0.125 mM each) and incubated for 5 more min at 37°C. The tubes were then transferred to room temperature and to each sample 40 µl of ligase mix (790 µl of

deionized water, 100 µl of 10 x ligase buffer, 100 µl of 50% PEG, 10 µl of 100 mM DTT, 5 units of T4 DNA polymerase) was added, mixed and incubated at room temperature for 1 hr. Twenty fine µl ligation mixture was used for transformation. Thirty transformants from each time point were double digested with *Eco*RI and *Hind*III for checking the sizes of deleted DNAs.

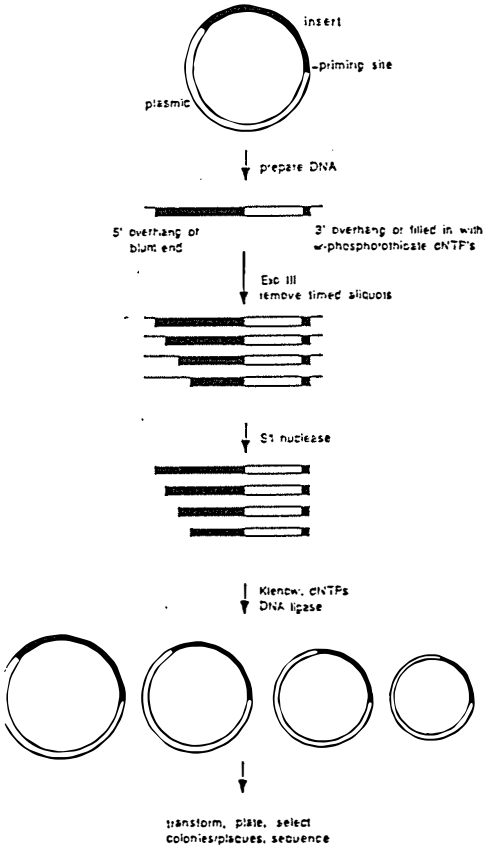


Fig. 2-3 A diagram of *Exo*III deletion of DNA.

2.5.14 DNA sequencing

2.5.14.1 T7 DNA polymerase sequencing reaction

T7 DNA sequencing system from Promega was used for sequencing cDNAs, which was according to Tabor and Richardson (1987). 4 µg (approximately 2 p moles) of supercoiled plasmid DNA dis-

solved in 18 µl of deionized water, then mixed with 2 µl of 2 N NaOH and incubated at room temperature or 85°C for 5 min. The solution was neutralized by adding 8 µl of 5 M ammonium acetate (pH 7.5). DNA was precipitated as described, and resuspended in 6 µl of distilled water, which mixed with 2 µl of 5 X T7 sequencing buffer, 2 µl of T7 or SP6 promoter primer (approximately 12-24 ng of 20 mer). The annealing reaction was done by incubation at 37°C for 15 min. To each reaction, the following reagents were added and mixed: 1 µl of DTT (100 mM), 2 µl of diluted labelling mix, 0.5 µl of [α-^{35}S]dATP, 2 µl of diluted T7 DNA polymerase (T7 DNA polymerase was diluted to 1.5 U/µl in ice cold 1 X TE buffer just before its use), and incubated at room temperature for 5 minutes. While the label reaction was incubated, 4 microcentrifuge tubes T, C, G and A were labelled, and 2.5 µl of the appropriate nucleotide mix were added to each of the four tubes. These tubes were stored at 4°C or on ice, and pre-warmed at 37°C for 1-2 minutes just before completion of the labelling reaction. At the end of the labelling reaction, the labelling mixture (3.5 µl) were immediately added to each of the nucleotide tubes and continued incubating at 37°C for 5 min. The reactions were stopped by adding 4 µl of stop solution to each tube. The samples were heated at 85°C for 5 min before loading the samples onto a sequencing gel. One - two µl of each reaction was usually loaded to each lane. The order of 4 lanes for one reaction was TCGA.

2.5.14.2 Polyacrylamide-urea gel electrophoresis

The glass plates were cleaned and dried. The surface of the small plate was treated with dimethyldichlorosilane solution (BDH, UK), rinsed with 95% ethanol and air-dried. The plates were assembled as a gel sandwich use, which was 38.5 cm x 31.0 cm x 0.4 mm. A 6% Polyacrylamide gel/urea sequencing gel was used, which was prepared by mixing following

reagents: 15 ml of 40% (w/v) (acrylamide: bisacrylamide; 19:1), 50 g of urea, 10 ml of 10 x TBE (121.1 g/l Tris base, 55 g/l boric acid, 7.4 g/l Na_2EDTA, pH 8.3), 35 ml of deionized water, 1 ml of 10% ammonium persulphate, and 10 µl of TEMED. After the gel was poured, the flat edges of two sharkstooth combs were inserted between the plates to a depth of 3-5 mm below the short plate. After the gel was polymerized, the combs were removed and gel sandwich was assembled to the sequencing apparatus Model S2 (BRL, USA). Upper and lower reservoirs were filled 1 x TBE, approximately 500 ml each. After the gel was pre-run at 65 W for 1 hr, the gel top was thoroughly rinsed with the running buffer before two sharkstooth combs were inserted until they just went into gel about less than 0.5 mm. 1-2 µl of sample were loaded immediately into the wells in the order of T, C, G and A after incubated at 85°C for 5 min and stored on ice. The power setting was around 65 W (about 45 mA x 1600 V). After the electrophoresis was finished, the gel was removed from the sandwich, soaked for 15 min in a fixing solution (5% methanol and 5% acetic acid), dried and analyzed by autoradiography.

2.5.15 Sequence analysis

DNA and peptide sequence analysis was performed using a software package developed by Genetics Computer Group (GCG), Madison, Wisconsin, USA.

CHAPTER 3. PURIFICATION AND CHARACTERIZATION OF TAMARILLO LECTINS

3.1 Introduction

As discussed in Section 1.3, solanaceous lectins are hydroxyproline rich glycoproteins which specifically bind to β(1,4) oligomers of N-acetylglucosamine. Although much work has been done on lectins from tomato, potato and *Datura* seed, little is known about the generic distribution and biochemical properties of other solanaceous lectins. This study aimed to screen and characterize new lectin(s) from solanaceous plants which may have potential economic value to New Zealand.

3.2 Results and discussion

3.2.1 Lectin screening

Cyphomandra species which were available in New Zealand and other plants of the Solanaceae family such as eggplant were screened for the presence of agglutinating activity using rabbit red blood cells. All the fruits from *Cyphomandra* species which were screened showed strong haemagglutinating activities at levels comparable to or even greater than that of tamarillo fruits (*C. betacea*). The species tested were: *C. betacea* (red tamarillo), *C. betacea* (yellow tamarillo), *C. diploconos, C. corymbiflora* subsp *mortoniana, C. cajanumensis* (casana) [for the cytogenetics of the tamarillo and its wild relatives, see Pringle, 1990]. The strong haemagglutination caused by the crude PBS extracts of these fruits could be inhibited by the β(1,4) linked trimer or tetramer of N-

acetylglucosamine or chitin hydrolysate, indicating that these species contained abundant lectins, which have not been previously reported. These new lectins could be easily isolated by a chitin affinity chromatographic method described below, which was used successfully in the isolation of lectins from *C. betacea* (red tamarillo). It would be expected that other species of this genus might also contain lectins.

3.2.2 Isolation of tamarillo lectins

Agglutination activity as indicated by haemagglutination and carbohydrate inhibition tests was only detected in the tamarillo fruit. There were no detectable activities in the flowers, leaves, stems or roots of *Cyphomandra betacea* (data not shown). This suggested that the expression of lectin activity is developmentally regulated and organ-specific.

For the isolation of tamarillo lectins, the procedure of chitin affinity chromatography followed by ion exchange was used. All the lectin activity from the extract of 400 g of tamarillo fruits was bound to the chitin column. No agglutinating activity was detected after several passages of the lectin extract on the chitin column. Lectin activity was eluted with 5 ml of chitin hydrolysate solution (Fig.3-1). The eluate was fractionated by ion exchange chromatography with a salt gradient, giving one sharp peak A at 0.37 M NaCl and one broad peak B at less than 0.19 M NaCl (Fig.3-2). Fractions from both peak A and B showed haemagglutination activities. Samples from these fractions were analyzed in a SDS-PAGE gel, showing a major protein band from peak A and several larger molecular weight bands for peak B (Fig.3-3). The lectin for peak A and peak B are designated CBL1 and CBL2

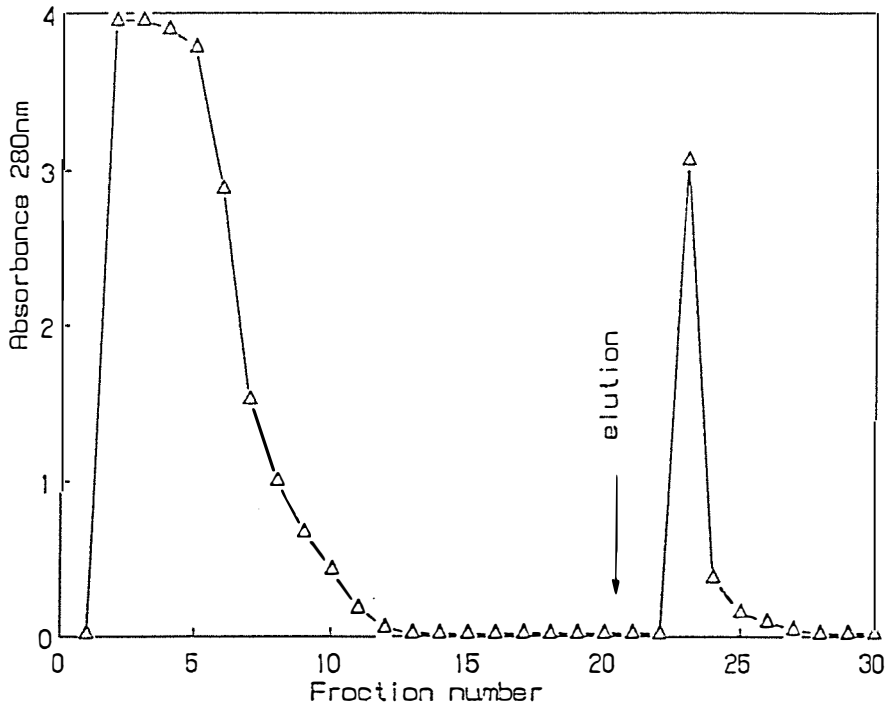


Fig. 3-1 Chitin affinity chromatography of lectins from tamarillo fruits (400 g). After ammonium sulphate precipitation, lectin sample was dissolved in buffer B and applied to a chitin affinity column. After washing with buffer B until absorbance at 280 nm dropped to zero, elution was done by applying 5 ml of chitin hydrolysate solution. Fractions of 10 ml each were collected.

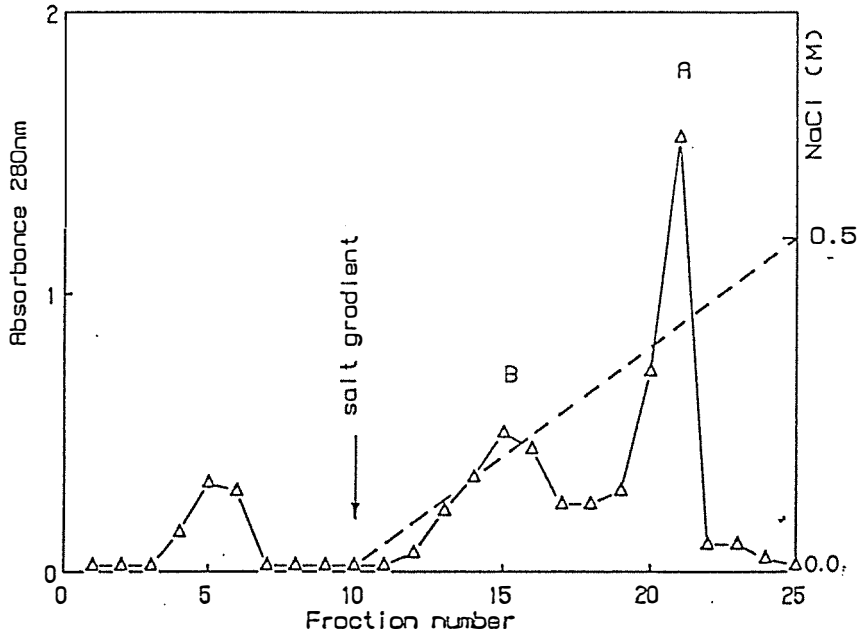
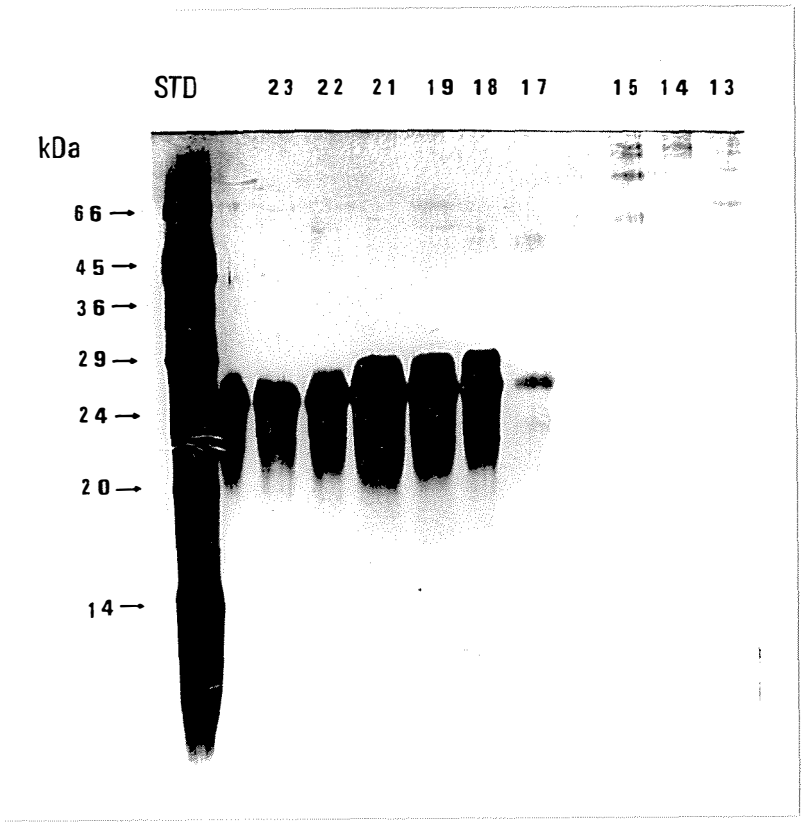


Fig. 3-2 Cation-exchange chromatography of tamarillo lectins on a S-Sepharose column. After chitin affinity chromatography, lectin sample was dialysed against Buffer C and applied to the S-Sepharose column. Lectin activity was eluted with a salt gradient. Fractions of 2 ml each were collected and subjected to protein electrophoresis (Fig.3-3).

Fig. 3-3 SDS/PAGE of tamarillo lectin samples from fractions of the S-Sepharose column. The numbers at the top of each lane correspond to the fraction numbers in Fig.3-2. STD, molecular weight standards. 10 μl of sample from each fraction was boiled with an equal volume of protein sample loading buffer with β-mercaptoethanol at 100°C for 5 min before applying to the 15% polyacrylamide gel. After electrophoresis, the gel was over-stained with a silver staining kit to examine the purity of samples. Fractions 13-15, 17-23 corresponded to lectin(s) from peak B and A respectively.


STD
23
22
21
19
18
17
15
14
13
kDa
66
45
36
29
24
20
14

respectively. Therefore, CBL1 was found to contain one single band with molecular weight about 25 kDa. CBL2 may contain more than one lectin with molecular weight of at least 50 kDa. CBL2 was not further characterized. Immunodiffusion tests of CBL2 did not produce a precipitin line with the anti-CBL1 serum (see Chapter 4). Thus, CBL1 and CBL2 appeared to be different molecular species.

Crystals of CBL1 could be readily formed in the purified lectin solution, indicating these purification procedures yielded homogeneous CBL1, which could be further studied in order to determine its three-dimensional structure.

Alternatively, CBL1 could also be purified by gel filtration chromatography at pH 3.6 and pH 7.2. The chromatography profile at pH 3.6 is shown in Fig.3-4. Fractions containing CBL1 were combined and freeze-dried. The CBL1 sample was then dissolved in 10 ml of buffer D (pH 7.2), subjected to gel filtration at pH 7.2. A single peak was obtained (data not shown). CBL1 sample purified by this approach also gave one single band on SDS-PAGE gel with molecular weight of 25 kDa, and CBL1 could also be crystallized (data not shown). Since this method was very time consuming, the chitin affinity chromatography method was routinely employed to isolate CBL1.

CBL1 was found to be an abundant protein in tamarillo fruits, the amount of CBL1 in lectin crude extract calculated from Table 3-1 accounts for about 8 % (mg/mg) of total protein in the crude extract (Table 3-1). About 17.2 mg of CBL1 could be obtained from 400 g of fruits by the chitin affinity purification procedure (Table 3-1). Purified CBL1 had no chitinase activity, showing that there was no chitinase contamination in the purified CBL1 sample (data not shown).

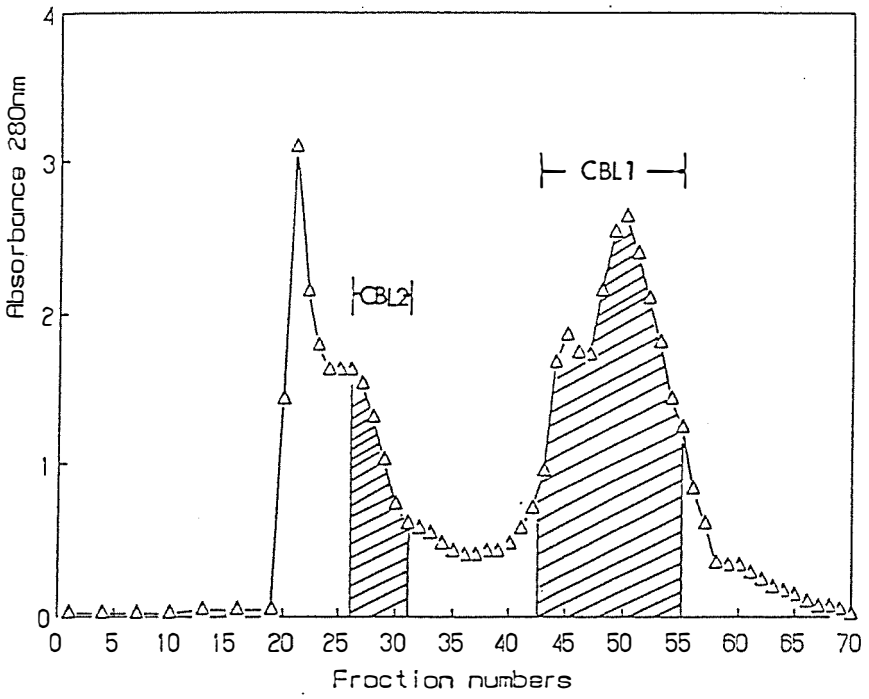


Fig. 3-4 Gel filtration chromatography of lectins from tamarillo fruits (400 g). After ammonium sulphate precipitation, lectin sample was dissolved in Buffer C (pH 3.6) and applied to a Sephacryl S-200 column equilibrated with Buffer C. Fractions of 5 ml each were collected. Shaded area had haemagglutination activity.

Table 3-1 Purification of tamarillo lectins. Starting material was 400 grams of ripen fruits.

Steps	Protein	Units	Specific Units	Yield
	mg	U x 10^6	U/mg x 10^3	%
Extract	789	4.5	5.7	100
$(NH_4)_2SO_4$) fractionation	160	3.3	20.6	75
Chitin	24	1.2	50.0	27
S-Sepharose	17.2 CBL1	0.7	40.7	-
	5.6 CBL2	0.3	53.6	-

3.2.3 Molecular size of CBL1

CBL1 was shown to be 25 kDa in size on SDS-PAGE (15%) gels after having been boiled with β-mercaptoethanol in the protein sample buffer (Fig.3-3) or without β-mercaptoethanol treatment (Fig.3-5). CBL1 was run on a Sephadex G75 gel filtration column at pH 3.6 and pH 7.2, and the elution profile was compared to a set of molecular weight standards run under the same conditions. The size of CBL1 was approximately 50 kDa under both conditions (Fig. 3-6). These data indicated that CBL1 had two subunits with identical molecular weight which were nonconvalently linked. The molecular size of CBL1 appeared to be smaller than other

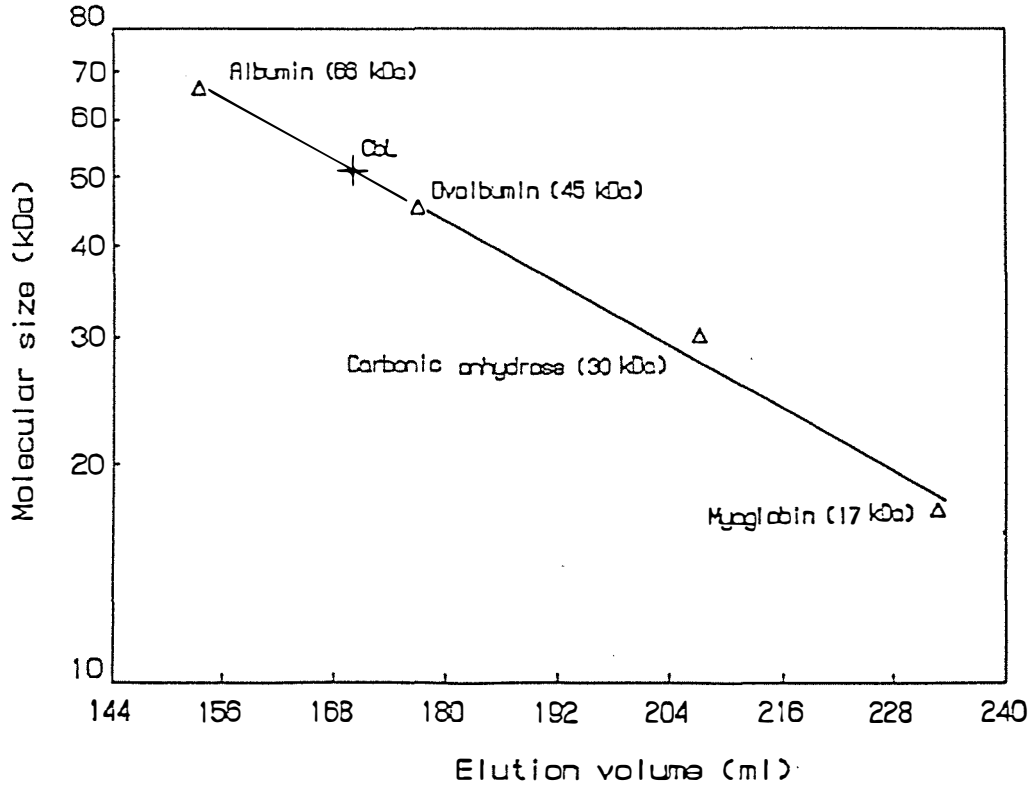
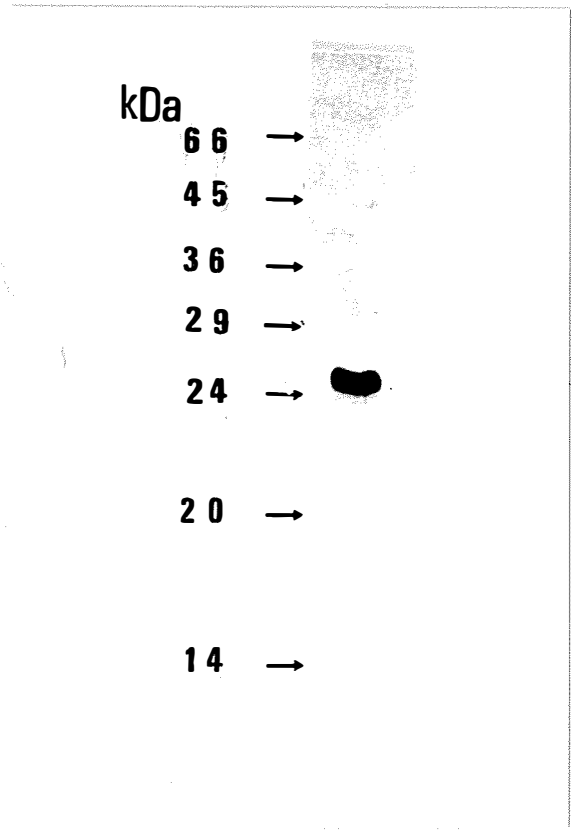


Fig.3-6 Molecular size determination of CBL1 by gel filtration chromatography on a Sephadex G75 column in either buffer C (pH 3.6) or buffer D (pH 7.2). Ten ml of 1 mg/ml purified CBL1 was applied to the column. Fractions of 5 ml each were collected.

Fig.3-5 Size determination of CBL1 by SDS-PAGE analysis. Five μl of lectin sample from fraction 21 (Fig.3-2) was boiled with an equal volume of the protein sample loading buffer without β-mercaptoethanol at 100°C for 5 min, before applying to the 15 % polyacrylamide gel. After electrophoresis, the gel was stained with a silver staining kit.

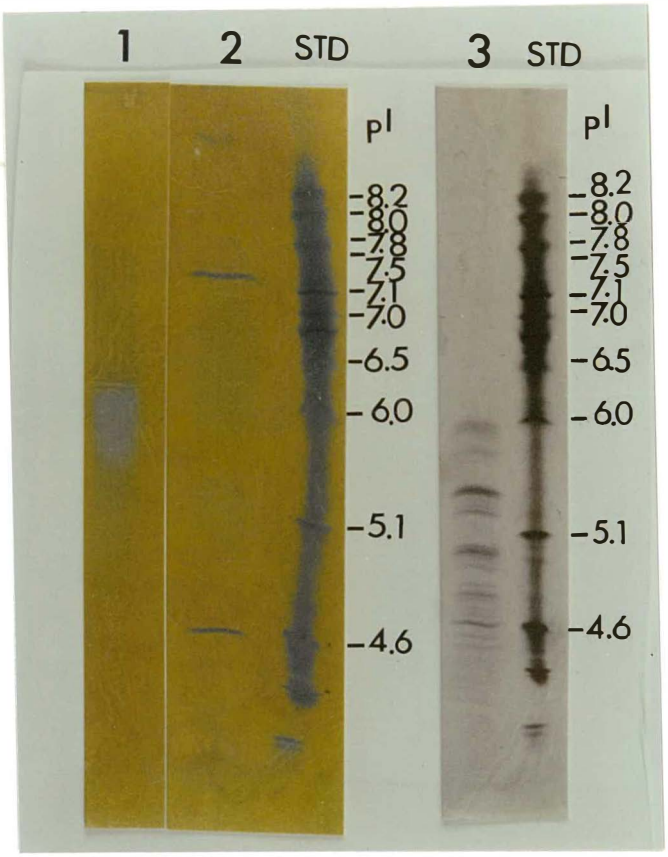

kDa
66
45
36
29
24
20
14

characterized solanaceous lectins, e.g. tomato lectin (LEL) which occurs as a single glycopolypeptide with a molecular weight of 71 kDa (Nachbar *et al.* 1980), *Datura* lectin (DSA) which is composed of nonidentical subunits (32 kDa and 28 kDa) joined by disulphide bonds (Kilpatrick and Yeoman, 1978; Crowley and Goldstein, 1981; Broekaert *et al.*, 1987) and potato lectin (STL) which is a dimeric glycoprotein (100 kDa) of two identical subunits which are nonconvalently linked (Allen and Neuberger, 1973).

3.2.4 Isoelectric points of tamarillo lectins

CBL1 purified from chitin affinity chromatography could not be focused on isoelectric focusing (IEF) gels, but gave a diffuse band ranging from pH 6.2 to pH 5.6 (Fig. 3-7, lane 1); whereas, CBL1 sample purified from gel filtration chromatography on a Sephacryl S-200 column gave two main bands corresponding to pH 5.4 and 5.0, and several minor bands ranging from pH 6.0 and 4.6 (Fig. 3-7, lane 3). The reason that CBL1 purified by chitin affinity could not be focused is not known, but may be due to its aggregation under electric field. CBL1 purified by gel filtration showed several bands on IEF gel, which could be due to the possible oxidation of its thiol group after freeze-drying processes during its isolation by gel filtration chromatography, or due to the presence of isolectins of CBL1. The discrepancy between the behaviours of CBL1 purified by the two different methods on IEF is unexpected, particularly since the CBL1 samples were identical by SDS-PAGE gel analysis. CBL1 purified by both methods also showed an ill-defined band at the top of polyacrylamide gel (9 % acrylamide) under non-reducing conditions (data not shown), which is similar to the findings on tomato lectin (LEL) (Merkle and Cummings, 1987a) where LEL was ill-defined at the top of gels under non-reducing conditions. This data suggests that CBL1 could be

Fig. 3-7 Isoelectric focusing of CBL1 and CBL2 samples. Lane 1. CBL1 purified by chitin affinity chromatography (about 5 μg); Lane 2. CBL2 purified by chitin chromatography (about 5 μg); Lane 3. CBL1 purified by gel filtration (about 5 μg); STD, standards.


1
2
STD
3
STD
pI
8.2
8.0
7.8
7.5
7.1
7.0
6.5
6.0
5.1
4.6
pI
8.2
8.0
7.8
7.5
7.1
7.0
6.5
6.0
5.1
4.6

aggregated under the electric field. From overall consideration of the data, it could be concluded that the pI value for CBL1 may fall within a range from 6 to 4.6. From this preliminary data, CBL1 is concluded to be an acidic lectin.

CBL2 sample gave two sharp bands at pH 7.3 and 4.6 (Fig. 3-7, lane 2). It is possible that the pI value for CBL2 was 4.6, since CBL2 was eluted first from an S-Sepharose column (Fig. 3-2) at 0.19 M NaCl (pH 3.6), whereas CBL1 showed a higher pI value and eluted at 0.37 M NaCl (pH 3.6). Thus, CBL2 could be readily purified by chromatofocusing techniques.

3.2.5 Carbohydrate specificity

Over 50 carbohydrates of various kinds were tested. These included mannose, glucose, galactose, N-acetylgalactosamine, N-acetylglucosamine, sialic acid, L-fucose or D-fucose and various oligomers or polymers. Oligomers of β(1,4) linked N-acetylglucosamine were the only carbohydrates showing inhibitory effects on CBL1 induced haemagglutination. CBL2 induced haemagglutination was also inhibited by the same oligosaccharides (Table 3-2). The minimum concentrations of

Table 3-2 The minimum concentration of sugars required for complete inhibition of 8 haemagglutination units.

Carbohydrate	LEL[a]	CBL1	CBL2
N-acetylglucosamine	>250 mM	>250 mM	>250 mM
N,N'-diacetylchitobiose	>15 mM	>30 mM	>15 mM
N,N',N''-triacetylchitotriose	0.6 mM	>18 mM	0.6 mM
N,N',N'',N'''-tetraacetylchitotetraose	0.3 mM	2.3 mM	0.3 mM

[a] Tomato lectin

N,N',N'',N"'-tetraacetylchitotetraose for the inhibition of CBL1 and LEL haemagglutination activity [8 haemagglutination units (HU)] were 2.3 mM and 0.3 mM respectively. The minimum concentrations of N,N',N''-triacetylchitotriose for the inhibition of CBL1 haemagglutination activity (8 HU) was less (18 mM) than that of LEL (0.6 mM), indicating a difference between the two in terms of sugar binding strength. CBL1 induced-haemagglutination could not be inhibited by N-acetyllactosamine and sialic acid which were inhibitors of DSA (Crowley *et al.*, 1984) and wheat germ agglutinin (WGA, a chitin binding lectin) (Wright, 1980a), respectively. It was thus demonstrated that the carbohydrate binding property of CBL1 differs in detail from those of other characterized solanaceous lectins and WGA, although they all bind to oligomers of N-acetylglucosamine. It should be noted that CBL2 showed a similar inhibitory profile in respect to carbohydrates as LEL (Table 3-2).

3.2.6 Stability of CBL1

Purified CBL1 was found to be an extremely heat stable protein, particularly at high concentrations. For example, the agglutinating activity of CBL1 at 0.5 mg/ml (256 haemagglutination units/10 µl) in water remained unchanged after incubation in a 100 °C heating block for 12 hours, the longest period tested. Repeated freeze-drying processes lowered its heat resistance, for instance, after CBL1 had undergone four freeze-drying processes, or if CBL1 was purified by gel filtration (with several freeze-drying processes) about 50 % of agglutination activity of CBL1 (0.5 mg/ml) was lost during the first 5 min of incubation at 100°C, and 75 % was lost during the first 10 min. At a lower concentration, CBL1 showed less heat resistance as shown in Table 3-3. About half of the activity of CBL1 at 8 µg/ml was lost during the first 5 min of incubation at 100°C. CBL1 also

Table 3-3 The effect of heat[a], the extremes of pH[b] and EDTA[b] on CBL1 haemagglutination activity.

Treatment	Time	Activity compared to control
100 °C	5 min	75 %
	10 min	50 %
	15 min	<25 %
90 °C	5 min	100 %
	10 min	75 %
	15 min	50 %
85 °C	5 min	100 %
	10 min	75 %
	30 min	50 %
75 °C	2 hrs	100 %
	4 hrs	50 %
	6 hrs	50 %
	20 hrs	0 %
pH 12.0 (0.05 N NaOH)	10 min	100 %
	30 min	100 %
	1 hr	100 %
	2 hrs	100 %
	4 hrs	<25 %
	6 hrs	<4 %
pH 2.4 (0.05 N citric acid)	10 min	100 %
	30 min	100 %
	2 hrs	100 %
	7 days	100 %
EDTA (100 mM)	-	100 %

[a] CBL1 at 8 µg/ml

[b] CBL1 at 0.5 mg/ml

showed resistance to extremes of pH, especially to low pH. For instance, at pH 2.4 the activity of CBL1 remained unchanged for 7 days, the longest period tested; at pH 12.0, the agglutination activity of CBL1 remained unchanged after 2 hrs, and 25% of its activity remained after 4 hrs. These properties may be due to a high number of intra-peptidyl disulphide bridges which could stabilize the subunit structure necessary for maintaining carbohydrate binding properties, since CBL1 contained many Cys residues (see below). CBL1-induced agglutination could not be inhibited by EDTA, suggesting that CBL1 might not require metal ions to exert its agglutinating property. All these properties resembled those of lectin from potato pericarp (Kilpatrick, 1980a), LEL (Kilpatrick, 1980b) and DSA (Kilpatrick *et al.*, 1978), but CBL1 showed much higher stability than these lectins.

3.2.7 Chemical composition

The amino acid composition of CBL1 is shown in Table 3-4, together with those of three well characterized solanaceous lectins from potato, tomato and *Datura*. There was a general resemblance of CBL1 to those known lectins in that they all contained abundant residues of Cys, Gly, Glx, Ser, Pro and Asx. Although CBL1 also contained hydroxyproline residues, the amount was much less than those of the other solanaceous lectins. In contrast, CBL1 contained an high amount of Cys residues. Carbohydrate analysis by a modified Dubois' method showed that CBL1 contained 24% carbohydrate which has not been characterized.

Table 3-4. Amino acid composition of CBL1 and other known lectins from Solanaceae.

AA	CBL1[a]	STL[e]	LEL[f]	DSA[g]	DSA subunit A[h]	DSA Subunit B[h]
Hyp	6.5	50	43	24	25	18
Asx	12.9	12	12	12	13	13
Thr	7.0[b]	14	13	10	12	13
Ser	19.8[b]	31	34	23	27	23
Glx	23.3	17	20	12	18	18
Pro	16.0	17	28	11	13	13
Gly	25.8	30	25	23	23	27
Ala	7.7	10	6	7	8	8
Cys	28.8[c]	26	17	22	26	31
Val	2.6	1	0	10	2	3
Met	0.9	1	7	1	0	0
Ile	1.0	4	2	2	0	1
Leu	4.8	3	0	3	2	3
Tyr	6.5	8	6	4	4	4
Phe	3.0	0.5	Trace	2	1	1
His	1.3	0	3	1	0	0
Lys	10.2	9	11	3	2	2
Orn	ND[d]	0.3-1	0	0	-	-
Arg	5.0	3	6	5	6	8
Trp	ND[d]	8	8	3	4	5

[a] The values, except where noted, are the means of those obtained with four different digestion periods (24, 36, 48 and 72 hrs) based on a molecular weight 25 kDa for the CBL1 subunit containing 24% carbohydrate.

[b] Determined by extrapolation to zero time for values obtained at the four different hydrolysis times.

[c] Determined as cysteic acid after the oxidation of CBL1 with performic acid.

[d] Not determined.

[e] STL, potato lectin, Allen et al. (1978)

[f] LEL, tomato lectin, Nachbar et al. (1980)

[g] DSA, Datura seed lectin, Desai et al. (1981)

[h] Broekaert et al. (1987)

3.2.8 Mitogenic activity

The purified CBL1 did not show mitogenic activity toward human lymphocytes (data not shown). Tomato lectin (LEL) also did not show mitogenic activity towards lymphocytes, but instead it suppressed mitogen-mediated transformation of chicken lymphocytes (Nachbar *et al.*, 1980) and was also antagonistic towards human lymphocyte transformation (Kilpatrick *et al.*, 1985). Interestingly, WGA, which is from the Gramineae family and has similar saccharide specificities to the solanaceous lectins, is also inhibitory to lymphocyte transformation (Greene and Waldmann, 1980; Barrett *et al.*, 1983). It would be interesting to know whether CBL1 has a similar antagonistic property.

3.3 Summary

A new lectin (designated CBL1) from red tamarillo fruit (*Cyphomandra betacea*) was identified and purified to homogeneity by chitin affinity chromatography followed by ion-exchange chromatography. CBL1 occurred in a dimeric form with 25 kDa subunit size. CBL1 was an abundant and stable protein. Amino acid composition analysis showed an abundance of Cys, Gly, Glx, Ser, Pro and Asx residues. Hydroxyproline residues were also present but in a lesser quantity than in other solanaceous lectins. The haemagglutination caused by CBL1 was most readily inhibited by N,N′,N′′, N′′′-tetraacetylchitotetraose. Yet to be studied is the possibility of any clinical significance or use for this lectin. CBL1 showed no mitogenic activity against human lymphocytes.

Because of its relatively high abundance, its organ-specific

expression and accumulation, the mechanisms for the control of the expression of this protein in fruits are of interest. In this regard, an attempt to study its localization in fruit tissue using immunocytochemical methods and the search for its cDNA sequence have been included in this study.

Other lectin(s) (CBL2) which was immunologically unrelated to CBL1 was also identified in tamarillo fruits. Their molecular sizes were larger than CBL1, but displayed the same carbohydrate specificity to CBL1.

High lectin activity was also detected in extracts of other fruits from the genus *Cyphomandra*. Species of this genus could serve as a new source of lectins. Further studies of lectins from this genus would be of interest in the analysis of the phylology of this group of lectins.

CHAPTER 4 IMMUNOLOGICAL RELATIONSHIPS AMONGST SOLANACEOUS LECTINS

4.1 Introduction

It is generally believed that solanaceous lectins are structurally related. This conclusion is based on their cross-reactive properties in double immunodiffusion tests. Solanaceous lectins from seeds of tomato, potato, *Nicandra* and *Capsicum* show lines of identity with *Datura* seed lectin (DSA) in reacting to anti-DSA serum, whereas non-seed lectins from tomato fruit juice, potato juice and potato tuber are cross-reactive (Kilpatrick *et al.* 1980). Anti-STL serum was able to form precipitin lines with either DSA or LEL, however, the antiserum which was absorbed with excessive glycopeptide of STL was unable to do so. Thus, it was suggested that cross-reaction among solanaceous lectins is due to their glycosylated portion (Ashford *et al.* 1982a). The double immunodiffusion tests in these reports were performed in agarose gels which did not contain inhibitors for lectin-glycoconjugante interactions. Such interactions of lectin and glycoconjugantes present in serum could theoretically give precipitin lines. The previous conclusion of close immunological relatedness of solanaceous lectins might then be in error. This study aimed to re-examine the immunological relationship of solanaceous lectins and to test whether the new lectins described here from *Cyphomandra* could cross-react with other solanaceous lectins.

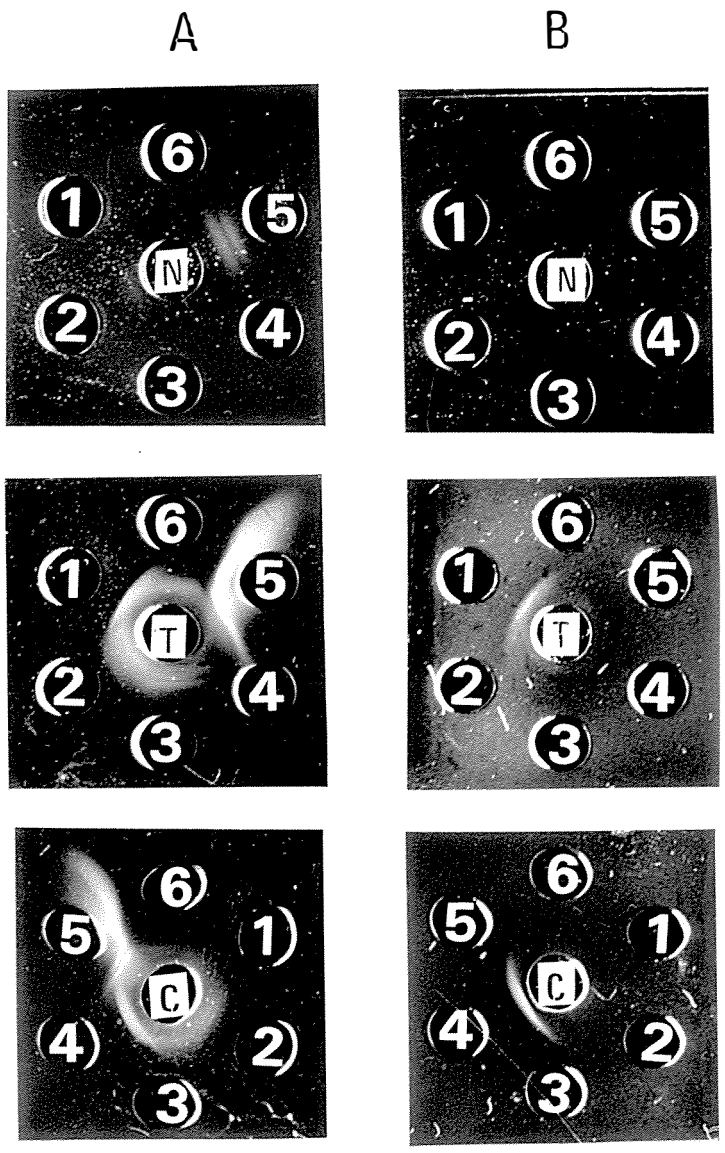
4.2 Results and discussion

As Fig 4-1 and Fig.4-2 show, in PBS buffered agarose plates (A panels), precipitin lines between following samples were observed: non-immune serum and CBL2; anti-LEL serum and LEL; anti-LEL serum and DSA; anti-LEL serum and STL; anti-LEL serum and CBL1; anti-LEL serum and CBL2; anti-LEL serum and WGA; CBL2 and WGA; anti-CBL1 serum and LEL; anti-CBL1 serum and DSA; anti-CBL1 serum and STL; anti-CBL1 and CBL1; anti-CBL1 serum and CBL2; anti-CBL1 serum and WGA; non-immune serum and fruit extract of *C. diploconos*; anti-LEL serum and fruit extract of *C. cajanumensis*; anti-CBL1 serum and fruit extract of *C. diploconos*. However, LEL and CBL1 were the only ones forming precipitin lines with their respective antisera in agarose plates (B panels) containing chitin hydrolysate. The hydrolysate would be expected to inhibit lectin-glycoconjugate interactions.

The lectin samples were also tested with non-immune rabbit serum. Only CBL2 and lectin extract of fruit of *C. diploconos* were shown to form precipitin lines with non-immune serum (A panels), but these lines were inhibited by chitin hydrolysate (B panels). This indicated the precipitin lines were due to interactions of the two lectin samples with glycoconjugantes in the serum.

This data suggested that: 1. Except those of CBL2 and from *C. diploconos*, precipitin lines formed were due to their reaction with antisera rather than components of non-immune sera. 2. Except those of LEL and CBL1, the formation of precipitin lines was due to the carbohydrate binding properties of the lectins with glycoconjugantes in the antisera, since they could be inhibited by the presence of chitin hydrolysate. 3. Precipitin lines of LEL and CBL1

Fig.4-1 Double immunodiffusion test I. Well N, non-immune serum (10 µl); well T, anti-LEL serum (10 µl); well C, anti-CBL1 serum (10 µl); well 1, LEL (5 µg); well 2, DSA (10 µg); well 3, STL (10 µg); well 4, (CBL1 5 µg); well 5, CbL2 (5 µg); well 6, WGA (5 µg). Panel A, agarose plates containing PBS; Panel B, agarose plates containing PBS buffered chitin hydrolysate.


A
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N
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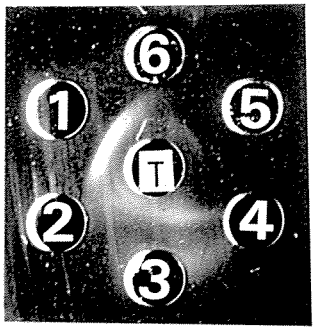
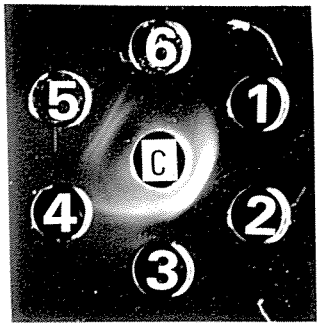
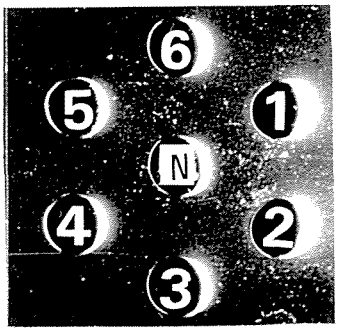
Fig.4-2 Double immunodiffusion test II. Well N, non-immune serum (10 µl); well T, anti-LEL serum; well C, anti-CBL1 serum (10 µl). Well 1, LEL (5 µg); well 2, CBL1 (5 µg); well 3, CbL2 (5 µg); well 4, fruit extract of *C. cajanumensis* (casana) [5 µg total protein, more than 256 haemagglutination units (HU)]; well 5, fruit extract from *C. diploconos* (10 µg of total protein, more than 256 HU); well 6, fruit extract from *C. corymbiflora* ssp *mortoniana* (10 µg of total protein, 128 HU). Panel A, agarose plates containing PBS; Panel B, agarose plates containing PBS buffered chitin hydrolysate.

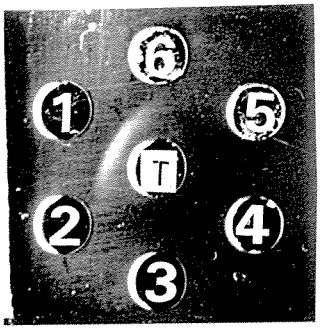
A

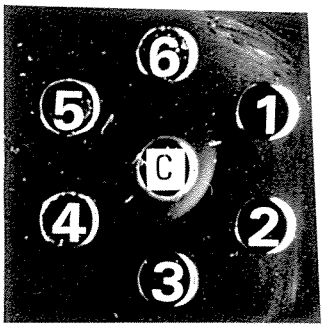




B







formed in PBS buffered agarose were due to both antigen-antibody reactions and their carbohydrate binding reaction as indicated by the lower intensities of their lines as shown in Fig.4-1 and Fig.4-2 (B panels) compared with those in Fig.4-1 and Fig. 4-2 (A panels).

The possibility was also considered that inhibition of precipitin line formation by chitin hydrolysate might be due to inaccessibility of antibodies to the carbohydrate binding sites, since they might be bound with sugars in the chitin hydrolysate. The possibility was rejected for the following reason: in free solution, the binding constants for immunoprecipitation of antigen-antibody are usually between 10^7 mol^{-1} to 10^9 mol^{-1} (Harlow and Lane, 1988), whilst those of carbohydrate binding constants of solanaceous lectins are generally only around 10^4 M^{-1}(Section 1.3.2). Thus, the antigen-antibody binding reaction would be expected to predominate over any lectin-ligand binding.

Data presented here then indicate that anti-LEL serum and anti-CBL1 sera recognised only their respective antigens, suggesting solanaceous lectins might not share similarities in their epitopes. This conclusion is at variance with the view that solanaceous lectins shared similar epitopes as already described (Kilpatrick *et al.*, 1980; Ashford *et al.*, 1982a; Desai *et al.*,1983). In their reports, immunological diffusion tests were performed in agarose gels that did not contain inhibitors of carbohydrate binding activity of the antigens. Thus, the precipitin lines observed in previous reports could be due to the following interactions: 1. interaction of antibodies in anti-sera with epitopes present on the surfaces of lectin molecules; 2. interaction of glycoconjugantes in antisera with carbohydrate binding sites of lectin molecules; 3. a combination of both. Ashford *et al.* (1982a) found that anti-STL serum from which antibodies

reacting with the glycopeptide portion of STL had been absorbed failed to show formation of precipitin lines with tomato and DSA, whilst the normal anti-STL serum could do. It was proposed that cross-reaction of LEL and DSA with anti-STL serum occurred at the oligoarabinose chains of the lectins (Ashford *et al.*, 1982a) or the cross-reactivity between STL, DSA and LEL depended on the integrity of the glycosylated regions of the lectins (Kilpatrick *et al.*, 1984; Kilpatrick, 1986). The present study does not support this view since it did not demonstrate the cross reactions of solanaceous lectin samples in agarose gels containing their carbohydrate ligands. However, their hypothesis could not be completely ruled out due to the possibility that anti-LEL and CBL1 sera might not have sufficient amount of highly specific antibodies recognising the carbohydrate moieties of these lectins.

Immunological double diffusion tests could provide some insights about the structural relationship among solanaceous lectins. However, due to the heterogeneous population of antibodies in the serum and variations in antigenicity of lectin in the individual rabbit, interpretation of diffusion data should be taken with care. Other techniques involving sequence analysis of these lectins seem more reliable ways of understanding their structural relationship.

4.3 Summary

Immunological double diffusion tests of solanaceous lectins in agarose gels containing their carbohydrate ligands showed no cross-reaction among the solanaceous lectins, as indicated by the absence of formation of precipitin lines. The epitope homologies among this class of lectins as suggested by the early reports have been questioned. The conclusion reached in

this thesis is that solanaceous lectins are not immunologically related.

CHAPTER 5 SUBCELLULAR LOCALIZATION OF CBL1 IN TAMARILLO FRUITS

5.1 Introduction

Immunocytological techniques have been used as powerful tools for the localization of macromolecules in plant materials. Highly specific antibodies are able to bind their antigens in plant tissue sections, and the antigen-antibody complex can be visualized by secondary antibodies which are conjugated with enzyme, fluorochrome or other markers (Hawes, 1988).

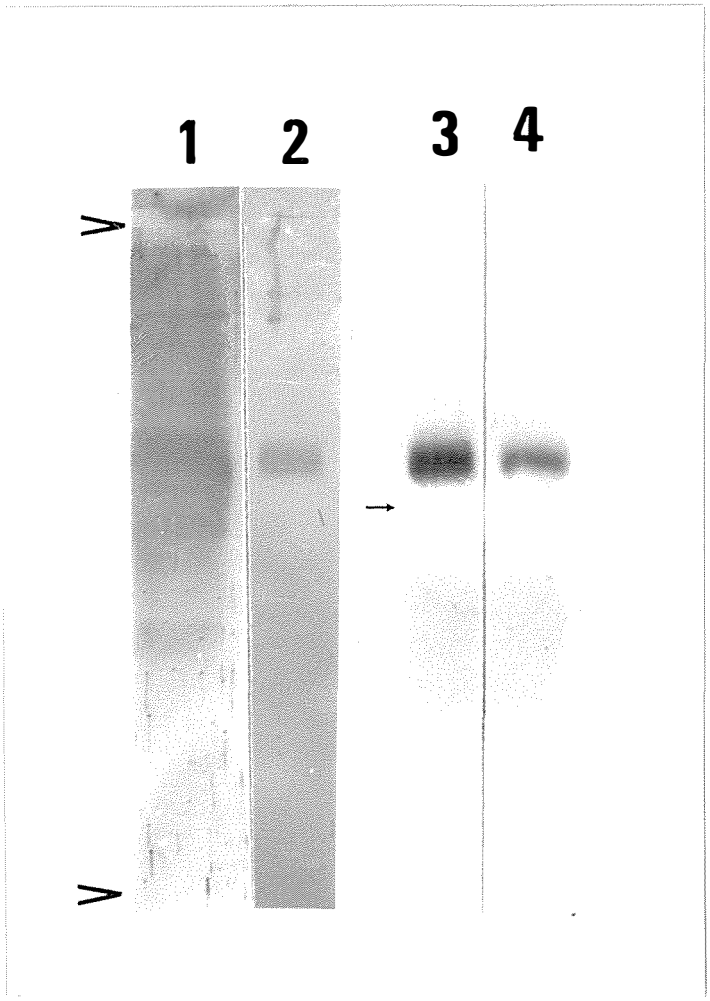
In this study, the secondary antibody was a biotinylated donkey anti-rabbit IgG, which could be recognized by a streptavidin-biotinylated phycoerythrin complex. Phycoerythrin, a red fluorescent phycobiliprotein from the algae *Rhodymenia palmata,* has an extinction coefficient of 1.96×10^6 M^{-1} cm^{-1} and a quantum efficiency of 0.8. The excitation peak occurs at 496 nm with a large Stokes shift to the maximum emission wavelength of 576 nm in the red segment of the spectrum. Sections which were labelled with phycoerythrin could be stored at room temperature in the dark for at least one year with minimal fading.

This study was aimed at determining the subcellular location of CBL1 in tamarillo fruits using the immuno-fluorescence microscopic technique described above.

5.2 Results and discussion

The antiserum used in this study was highly specific to the CBL1 as shown by western blotting (Fig.5-1). A single intensive band of 25 kDa (Fig. 5-1, lane 3) corresponding in

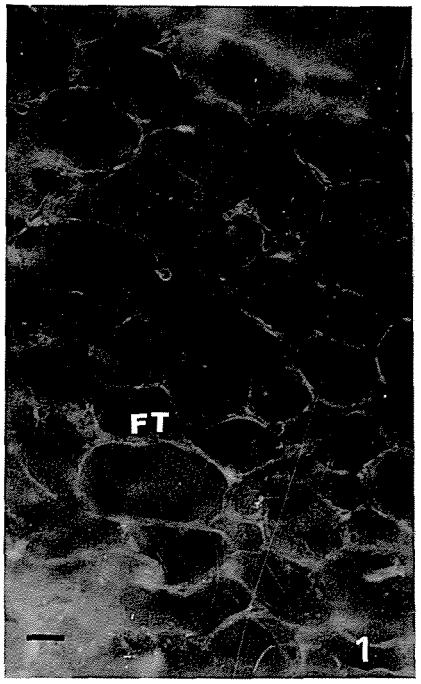
Fig. 5.1 Western blotting of purified CBL1 and tamarillo extract with anti-CBL1 serum. Lane 1, fruit extract (about 10 μg of protein); Lane 2, purified CBL1 (about 2 μg); Proteins were stained with colloidal gold (lanes 1,2). Lane 3, and 4 corresponded to anti-CBL1 serum reaction to proteins in lane 1 and 2 respectively after western blotting. The top of the sample slot and the dye front were marked by arrowheads. A faint band in lane 3 was marked by an arrow. For the total protein extract, fruit material was homogenized and boiled with the protein sample buffer at 100°C for 5 min before cell debris was removed by centrifugation 13,000 rpm for 3 min. Proteins were visualized after transfer from SDS-PAGE gels (15%) to nitrocellulose filters.

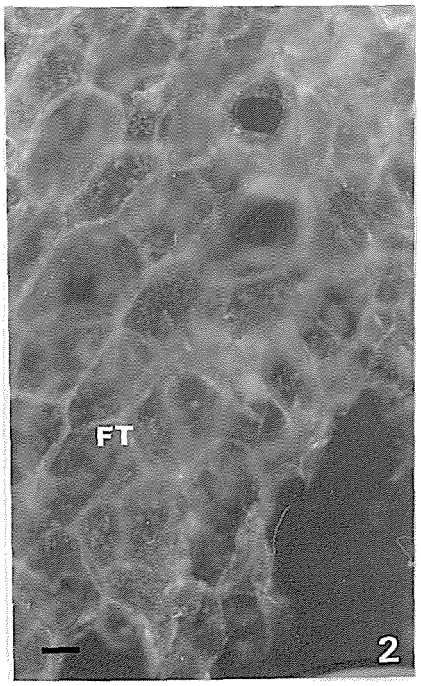

1
2
3
4

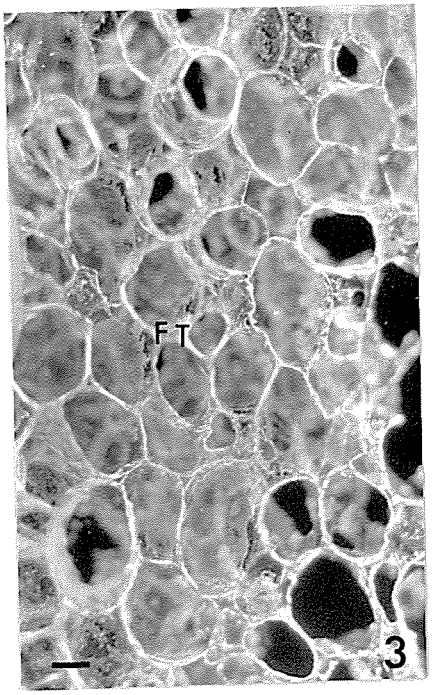
molecular weight to that of purified CBL1 (Fig. 5-1, lane 4) in the total protein extract was recognized by anti-CBL1 serum. Therefore, the intensive band in lane 3 corresponded to CBL1. To a much lesser extent, a faint band with a smaller size than CBL1 was seen on some of the blots, its position is marked by an arrow in lane 3 of Fig. 5-1, this faint band might be a degradation product of CBL1.

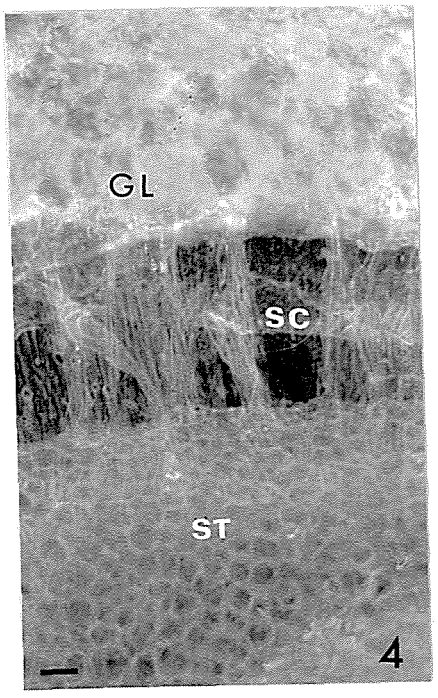
Anti-CBL1 serum was used to react with thin hand sections of young tamarillo fruits of approximately one month old (about 5 cm long, 3 cm in diameter). Such sections when incubated with the non-immune serum, or antiserum absorbed with excessive purified CBL1, or secondary antibody and phycoerythrin complex or just phycoerythrin showed low level fluorescence intensity [Fig. 5-2(2)], albeit higher than that of auto-fluorescence of sections incubated with BSA [Fig. 5-2(1)]. Incubation of sections with anti-CBL1 serum (1:20 in PBS-BSA), followed by biotinylated donkey anti-rabbit IgG (1:200 in PBS-BSA), and by streptavidin-biotinylated phycoerythrin complex (1:50 PBS-BSA) gave intense and specific labelling of cell walls of fruit tissues [Fig 5-2(3)]. Since fruit sections were not fixed, contents of some cells might be lost during incubation and washing steps as shown in [Fig. 5-2(3)], although most fruit tissue cells, when viewed in bright field, contained intracellular contents in the form of packed vesicles. Cell walls of these cells without contents were still intensely labelled, suggesting fluorescence was localized in the cell walls rather than inside the plasmalemma. There was little or no staining of cytoplasm, suggesting that CBL1 did not associate with the membranes of cell organelles. Fig.5-2(4) shows that fluorescence labelling appeared predominantly in fruit tissues rather than seed tissues, suggesting the CBL1 might be a fruit tissue specific lectin. It was however interesting to note that seeds were surrounded by gel-like material which was also labelled with fluorescence Fig. 5-2(4). In addition,

Fig. 5-2 Immunocytochemical localization of CBL1 using immuno-fluorescent techniques. 1. Control test, auto-fluorescence of sections which have not been incubated with serum or antibody; 2. Control test, fluorescence of sections incubated with anti-CBL1 serum which had been absorbed with excessive CBL1. 3. Fluorescence of fruit tissue sections incubated with anti-CBL1 antiserum. 4. Fluorescence of seed tissue sections incubated with anti-CBL1 serum. FT, fruit tissue; ST, seed tissue; SC, seed coat; GL, gel-like material. Magnification 200 x; Bar = 35 μm.


FT
1


FT
2


FT
3

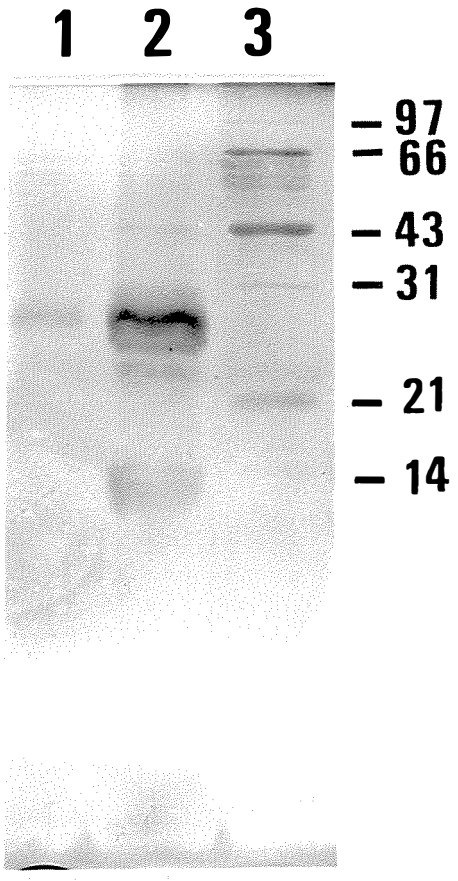

GL
SC
ST
4

two layers of extraordinary long cells (presumably seed coat) were also labelled with fluorescence, indicating the presence of CBL1. The origin of this seed coat associated accumulation is not known. It may originate as a testa product or alternatively it may be transported from the adjacent fruit tissues. A cell wall location for CBL1 suggests the possibility of at least a proportion of the lectin being mobile or subject to leaching from fruit tissues.

Tamarillo fruit juice was found to contain predominantly a protein with size 25 kDa (Fig. 5-3, lane 2) corresponding to that of purified CBL1 (Fig.5-3, lane 1). This predominant protein band could be recognized by anti-CBL1 serum (data not shown), also suggesting the extracellular location of this lectin, which might be secreted from the cells or dissociated from cell walls.

CBL1 was rich in Cys residues (Chapter 3), its localization in cell walls is consistent with the view that many extracellular proteins are Cys-rich proteins, which have disulphide bridges which stabilize the folded proteins (Thornton and Taylor, 1989). Since CBL1 is a chitin binding protein, its localization in cell walls is also consistent with the observation that chitin binding proteins have so far been detected predominantly in cell walls. Most potato lectin (STL) is tightly and nonconvalently bound to the cell walls (Casalongué and Pont Lezica, 1985), although an earlier report by Muray and Northcote (1978) suggested that this lectin was associated with root membranes, but the authors did not examine cell walls for the presence of lectin. Immuno-fluorescent study of the localization of *Datura* seed lectin (DSA) by Jeffree and Yeoman (1981) indicated it was associated with the plasmalemma and the membranes of cell organelles, however, the lectin sample used for antigen was not homogeneous (Kilpatrick and Yeoman, 1978). Broekaert *et*

Fig. 5-3. SDS-PAGE analysis of tamarillo fruit juice (15 % gel). Lane 1, purified CBL1 (about 1 μg). Lane 2, fruit juice (5 μl). Lane 3, protein standards. Fruit juice was boiled with protein sample buffer for 5 min after it was centrifuged at 13,000 rpm for 3 min.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al.(1988), using a highly specific immunoglobulin fraction purified on a DSA-sepharose 4B column, showed that most of the lectin is associated with cell walls of the seed coat and seed epidermis. Tomato lectin (LEL) was located predominantly in the locular fluid of ripe tomatoes (Merkle and Cummings, 1987a). Wheat germ agglutinin (WGA) was located at the cell wall-protoplast interfaces and in protein bodies (Mishkind *et al.*, 1982). In the latter sites, WGA could be involved in the post-translational process (Mansfield *et al.*, 1988). Wheat germ lysozyme, a chitin binding protein, also associates with cell walls (Audy *et al.*,1988).

The exact *in vivo* role of these chitin binding lectins is still unknown. A chitin binding lectin from stinging nettle rhizomes inhibits the growth of several pathogenic fungi containing chitin as a cell wall component (Broekaert *et al.*, 1989). DSA can be preferentially released during early imbibition of *Datura* seed (Broekaert *et al.*, 1988), and STL is also secreted upon imbibition of potato tuber slices (Casalongué and Pont Lezica, 1985). Potato tuber virus X can induce the expression of different genes encoding potato isolectins (Scheggia *et al.*, 1988). The proteins encoded by potato wound-induced genes show extensive homologies with WGA and chitinase (Stanford *et al.*, 1989). They could be members of the potato lectin gene family. Based on these studies and their localization in plant cell walls, it is possible that these lectins or chitin binding proteins could have a defence role *in vivo*. In this respect, CBL1 could be involved in the protection of the seed from external pathogens containing chitin in their outer walls. In addition, CBL1 could also be involved in chemical transduction or transportation between fruit tissues or seed tissues in order to facilitate seed development.

5.3 Summary

CBL1 was localized in cell walls of fruit tissues and also found in large quantity in the tamarillo juice. CBL1 might play a defense or communication role for facilitating seed growth and development.

CHAPTER 6 SEQUENCE ANALYSIS OF TAMARILLO LECTIN (CBL1)

6.1 Introduction

As described in earlier chapters, CBL1 was found to be a very stable, abundant and developmentally-regulated extracellular protein. Thus, it was of interest to study its gene structure and expression at the molecular level. The general strategy used was as follows. A cDNA library representing the mRNA population was constructed using polyadenylated RNAs extracted from young tamarillo fruits. Putative CBL1 cDNA clones, which were identified by screening the library with anti-CBL1 serum, were then sequenced. Sequences of these cDNA clones were then compared to those of CBL1 tryptic peptides to confirm whether they encoded CBL1. Such CBL1 cDNA, once confirmed, could be used in the future as a probe for the isolation of CBL1 gene from a genomic library.

As a first attempt towards understanding the structure and expression of CBL1, this study was aimed at two areas.
1. Determination of several peptide sequences. This sequence information could be used for the synthesis of an oligonucleotide probe for the screening of a cDNA library and for the characterization of positive clones as described.
2. Construction of a λgt11 cDNA library of young tamarillo fruits, immuno-screening of this library with anti-CBL1 serum, and sequence analysis of positive clones.

λgt11 (Young and Davis, 1983a,b) is an expression vector that carries a copy of the *E. coli LacZ* gene, with a single *Eco*R1 cleavage site located 53 bp upstream of the translational termination codon of the *LacZ* gene (Fig.2-1). Up to approximately 7.2 kb of foreign DNA can be accommodated at

this site. Coding sequences inserted in the correct reading frame and orientation will be expressed to yield fusion proteins whose amino termini consist of β-galactosidase sequences and whose carboxyl termini consist of foreign polypeptides. Some of the fusion proteins will display antigenic epitopes that can be detected by their ability to react with specific antibodies. The genotype of λgt11 is shown in Table 2-1. λgt11 encodes for a temperature-sensitive repressor (*cIts*857) and an amber mutation (*S*am 100) in the lysis gene. It can therefore form plaques on a lawn of *E. coli* cells that carry the amber suppressor *SupF* and can give rise to lysogens at temperatures at which the repressor is active. Fusion proteins carrying foreign antigenic epitopes can be detected by screening plaques with an immunological probe. This lambda phage is usually plated at 42°C on *E.coli* Y1090*hidR* (see relevant genotype in Table 2-1). After 4 hours, the plates are shifted to 37°C and overlaid with nitrocellulose filters impregnated with isopropylthio-β-D-galactopyranoside (IPTG), which inactivates the *lac* repressor and induces expression of the fusion protein.

Proteins released following the lysis of cells in plaques are immobilized onto nitrocellulose filters by direct contact with these plaques. The immobilized proteins are probed with primary antibodies. Antibody binding is revealed in a second step by probing the filters with secondary antibodies, which can be detected with immunochemical methods (Sambrook *et al.*, 1989, Huynh *et al.*, 1985). In this study, primary antibody binding was revealed in a second step by probing the filters with a goat anti-rabbit IgG conjugated with alkaline phosphatase, which recognized rabbit anti-CBL1 IgG, and converted *in situ* the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) into a dense blue compound by its alkaline phosphatase activity.

In this study, sequences of five peptides of CBL1 were determined and two cDNA clones, isolated by immunological screening of a tamarillo fruit cDNA library, were sequenced and analyzed.

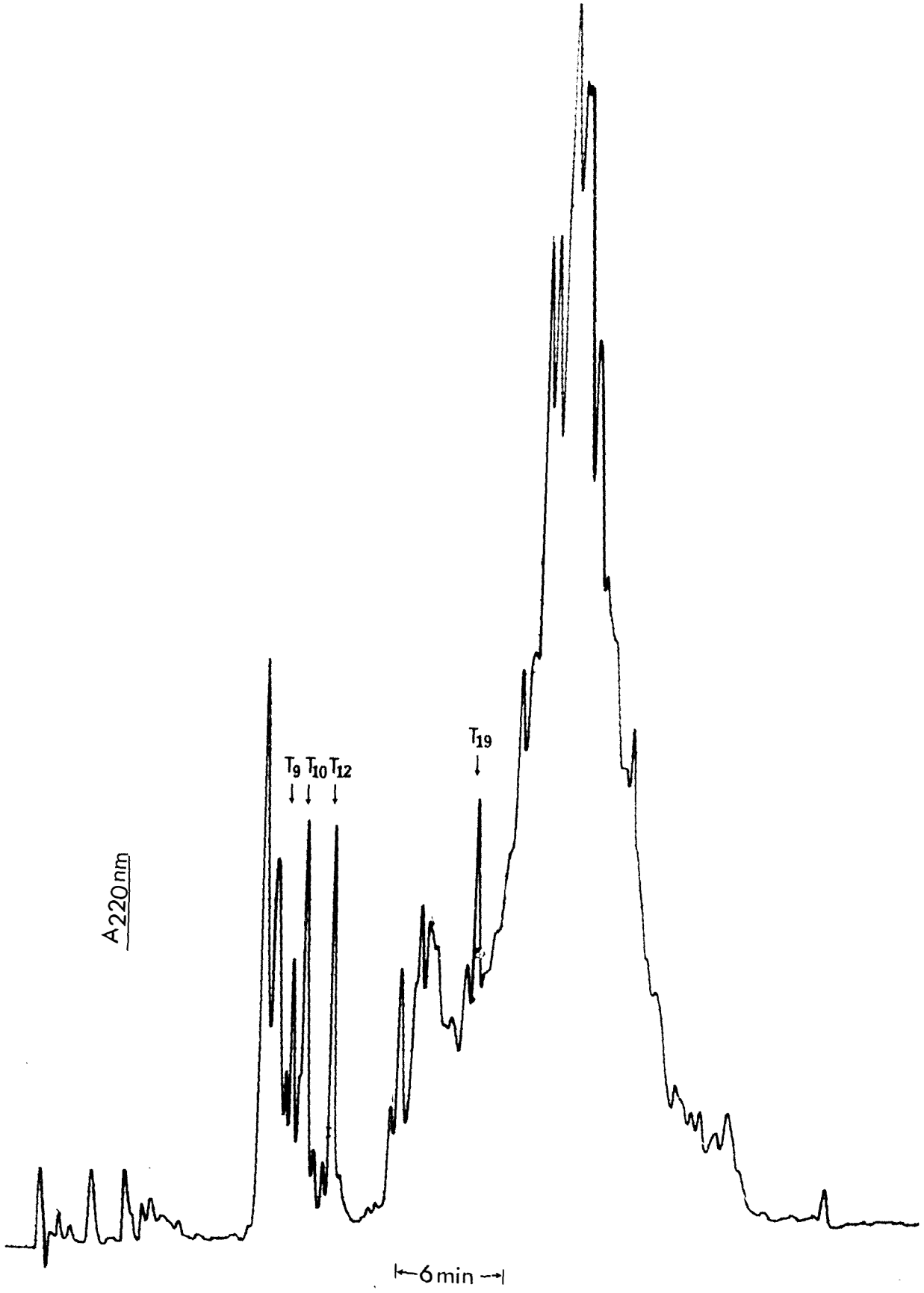
6.2 Results and discussion

6.2.1 Peptide sequences

CBL1 was denatured in guanidinium chloride and dithiothreitol (DTT), alkylated with 4-vinylpyridine before being digested with trypsin. Tryptic peptides were partially separated by high performance liquid chromatography (HPLC) (Fig.6-1). The peptide sequences from peak T9, T10, T12, T19 were determined as follows: T9, Ser-Gly-Cys-Gln-Ala-Asp-Gly; T10, Tyr-Gly-Cys-Gly-Ala-Asp-Gly-Arg; T12, Gly-Cys-Gln-Ala-Gly-Gly-Arg-Glu-Ser-Pro; T19, Ser-Gly-Cys-Gln-Ala-Asp-Gly. The N-terminal sequence of CBL1 was determined as Thr-Ser-Glu-Leu-Pro-Gln. The amino acid composition of these peptides was consistent with that of CBL1, e.g. abundance of Gly, Glx and Cys residues.

The peptide sequence of T12 showed some homologies with the Gramineae lectins such as WGA (Raikhel and Wilkins, 1987), barley root lectin (Lerner and Raikhel, 1989) and rice lectin (Wilkins and Raikhel, 1989)(Fig.6-2A). Tri-peptides such as Tyr-Cys-Gly, Ala-Gly-Gly, Gly-Cys-Gln as shown in the CBL1 peptide sequences were also found throughout the polypeptide of WGA (Raikhel and Wilkins, 1987). In addition, peptides T9, T10 and T12 also showed high homologies with WGA at the level of nucleotide sequences based on backtranslation of the protein sequences (Fig.6-2B). Despite a lack of sugar and hydroxyproline residues in WGA, as shown in Table 6-1, WGA

Fig. 6-1 Partial separation of a tryptic digest of valyl-CBL1 by HPLC. Peptides from peak T9, T10, T12 and T19 were sequenced.


T9 T10 T12
T19
A220nm
6 min

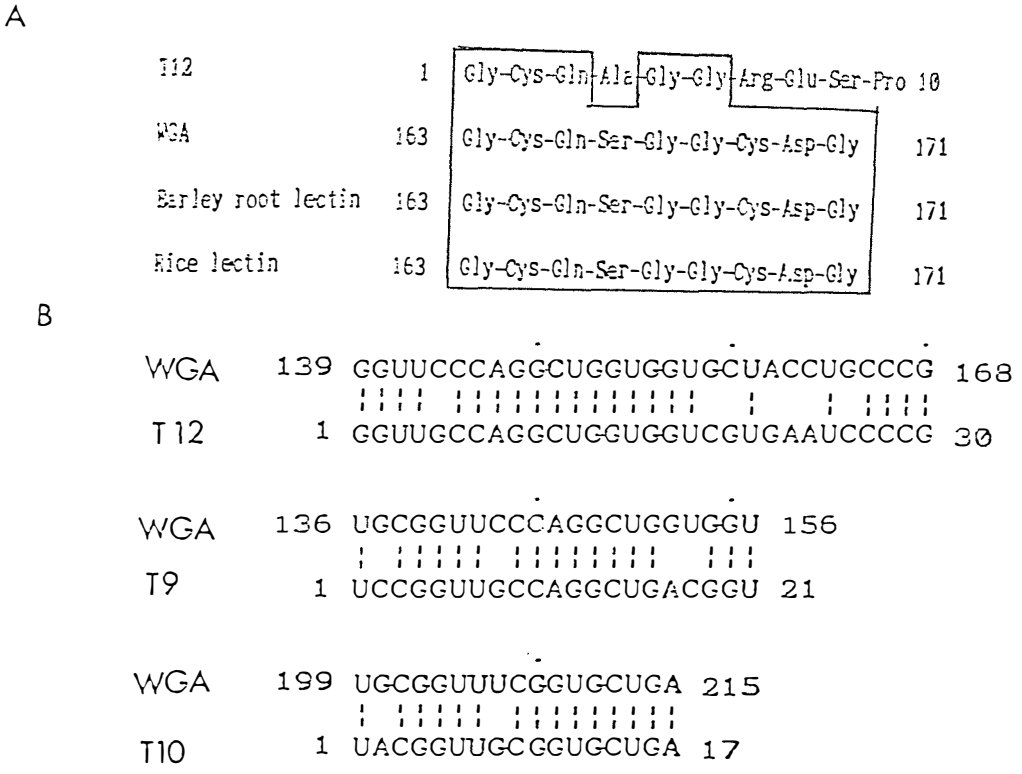


Fig. 6-2 Sequence homologies of CBL1 and WGA. A. Homologies (boxed) between T12 peptide of CBL1, WGA, and other related molecules. B. Homologies between nucleotide sequences of CBL1 peptides and WGA (Raikhel and Wilkins, 1987) based on the backtranslation of their protein sequences using a same frequency of codon usage (for enteric bacterial genes).

Table 6-1 Amino acid composition of CBL1 and WGA[a].

AA residues	CBL1	WGA	AA residues	CBL1	WGA
Hyp	6.5	-	Met	0.9	2
Asx	12.9	15	Ile	1.0	2
Thr	7.0	4	Leu	4.8	4
Ser	19.8	16	Tyr	6.5	7
Glx	23.3	15	Phe	3.0	2
Pro	16.0	6	His	1.3	2
Gly	25.8	42	Lys	10.2	6
Ala	7.7	10	Arg	5.0	4
Cys	28.8	32	Trp	ND	1[b]
Val	2.6	1			

[a] from Wright, 1984

[b] from Raikhel and Wilkins, 1987

ND not determined.

has a generally similar amino acid composition to CBL1, and both lectins bind to oligomers of β1,4 linked N-acetylglucosamine. Therefore, CBL1 could be evolutionarily related to WGA or other Gramineae lectins, since the Gramineae lectins also show specific binding to N-acetylglucosamine and its β(1,4) linked oligomers, and they are antigenically, biochemically and structurally related (Goldstein and Poretz, 1986; Poola, 1989; Stinissen *et al.*, 1983; Raikhel and Wilkins, 1987; Lerner and Raikhel, 1989; Wilkins and Raikhel, 1989).

6.2.2 cDNA library construction

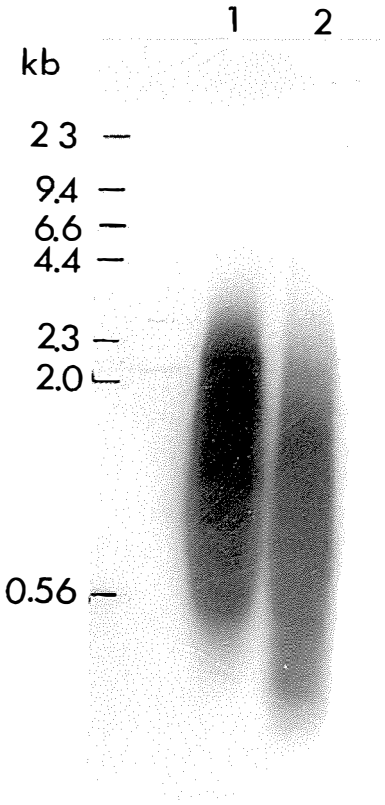
Poly(A^+) RNAs were isolated from young tamarillo fruits using an oligo(dT) cellulose affinity column, and were checked for their integrity using a wheat germ *in vitro* translation system. Autoradiography of SDS-PAGE gels of the *in vitro* translation products, indicated that proteins of a wide molecular weight range could be translated from the poly(A^+)RNA preparation. This suggests that purified mRNAs were intact (Fig 6-3).

Complementary DNA (cDNA) synthesis of these mRNAs was performed using AMV reverse transcriptase. From the incorporation assay, 23% of mRNAs were converted into the first strand of cDNA. 90% of the first strand cDNAs were converted into the second strand of cDNAs. A total of 336 ng of double stranded cDNA was synthesized from 1 μg of poly(A^+) RNAs. The first strand cDNAs and double stranded cDNAs were denatured and analyzed by alkaline gel electrophoresis and subsequent autoradiography. Size distribution of the first and second strand cDNAs was between 500 bp to 3000 bp (Fig. 6-4). The lower intensity of the second strand lane was due to a smaller amount of sample being loaded than that of the

Fig. 6-3 SDS-polyacrylamide gel (15 %) electrophoresis of *in vitro* translation products of poly(A^+) RNAs. Lane 1, poly(A^+) RNAs from tamarillo fruits (1 µg). Lane 2, globin mRNA (1 µg).


1
2

Fig. 6-4 Size distribution of the newly synthesized first strand cDNA and double stranded cDNA (ds cDNA). DNA samples were analyzed by alkaline agarose gel (0.8 %) electrophoresis followed by autoradiography. Lane 1, the first strand cDNA. Lane 2, ds cDNA.


1
2
kb
23
9.4
6.6
4.4
2.3
2.0
0.56

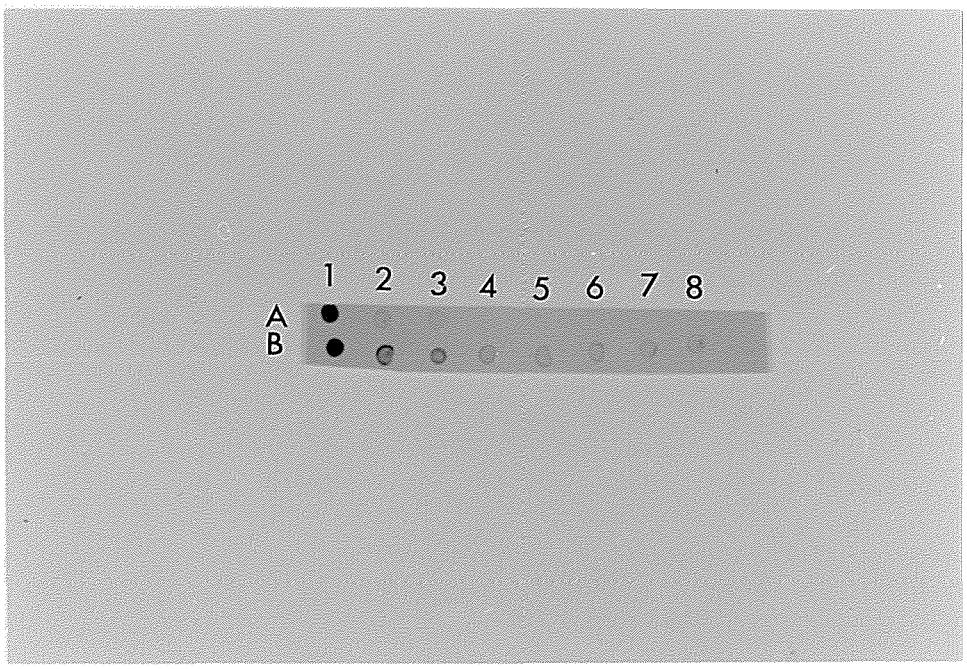
first strand. Thus, the synthesized cDNAs were in good quality in terms of the size range.

Double stranded cDNAs were then methylated, ligated to *Eco*R1 linkers, digested with *Eco*R1, and ligated to λgt11 arms and transfected to *E. coli* strain Y1090. Methylation of cDNAs was to prevent subsequent digestion of internal *Eco*R1 sites in cDNAs. The bacteria cells were mixed with soft agar or agarose which contained 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and IPTG, plated on LB plate. Recombinant λgt11 phages (with inserts of cDNA) were shown as white plaques, since the inserts would disrupt the *Lac*Z gene function which resulted in the inactivating of β-galactosidase which would otherwise react with the chromogenic substrate X-gal. The total number of recombinant plaques was 1.6 x 10^5. Half of these phages were amplified and stored either at -70°C or 4°C. Twelve white plaques were randomly picked and their insert sizes were examined, three of them were less than 500 bp, one of them about 3-4 kb, the rest were between 800 bp to 3 kb (data not shown). The cDNA library was thus considered to be reasonably good in terms of the wide range of insert sizes that it represented. Due to the abundance of CBL1, a cDNA library of this size would have a very good chance of containing cDNA inserts encoding CBL1.

6.2.3 cDNA library screening

Western blotting experiments showed that anti-CBL1 serum was highly specific to CBL1 (Fig.5-1). Dot blot analysis showed that anti-CBL1 antiserum was capable of detecting about 80 pg of denatured CBL1 in a dot 1.5 mm in diameter (Fig. 6-5). Thus, this sensitivity of antiserum should allow all but the most labile fusion proteins to be recognized (Sambrook *et al.*, 1989). Therefore, Anti-CBL1 serum was used to screen

Fig. 6-5 Dot blot analysis of CBL1 on nitrocellulose filters. Lane A, serial dilutions of CBL1 denatured in the SDS-PAGE loading buffer under reducing conditions. A1, A2, A3, A4, A5, A6, A7, and A8 correspond to the protein amount (per dot 1.5 mm in diameter) 0.8 ng, 80 pg, 50 pg, 40 pg, 24 pg, 16 pg, 8 pg and 0.8 pg. Lane B, serial dilutions of native CBL1. B1, B2, B3, B4, B5, B6, B7 and B8 correspond to the protein amount (per dot with 1.5 mm in diameter) 1.6 ng, 160 pg, 101 pg, 80 pg, 48 pg, 32 pg, 16 pg and 1.6 pg. Protein was visualized by the immunological screening method used for cDNA library screening.


1 2 3 4 5 6 7 8
A
B

the expressional cDNA library of young tamarillo fruits.

About 30,000 plaques from the unamplified and amplified library (15,000 each)(half of total number of recombinant phages) were screened. About 20 positive plaques were obtained. As shown previously, CBL1 was an abundant protein in tamarillo fruits, it was reasonable to speculate that the fruits might have relatively high copies of mRNAs which encoded CBL1. Thus the relative high frequency of positives was expected. Five of these positives were further purified to homogeneity, and their inserts were digested either with *Mlu*I or *Eco*R1. The positive recombinant phages X16, X58 and X60 contained cDNA inserts of less than 200 bp in size (data not shown), which might arise from fragments of mRNA encoded CBL1, or they may have similar epitopes to CBL1. cDNA insert sizes of positive recombinant phages X208 and X200 (Fig. 6-6) were around 500 bp and 900 bp respectively (Fig. 6-7). Since the molecular size of the CBL1 subunit was 25 kDa containing 24 % carbohydrate, its peptide size could be 19 kDa. Thus, CBL1 subunit size would be about 172 amino acid residues, which would require 516 bp for encoding. Therefore the X200 clone could be a candidate for encoding CBL1, and the X208 clone could partially encode CBL1.

6.2.4 DNA sequencing and sequence analysis

cDNA inserts of X200 and X208 were subcloned to the *Eco*R1 site of pGEM-4Z plasmid. The X200 insert was also subjected to *Exo*III digestion to shorten its size for sequencing (Fig.6-8). The two sequences were preliminarily determined as shown in Fig.6-9 and 6-10, which were not all checked by sequencing the opposite strand. Since compression was not problematic, the sequence information should be accurate. Neither cDNA sequences contained apparent polyadenylation

Fig. 6-6 Immunological screening and identification of recombinant phages X208 and X200 on nitrocellulose filters. 1. Homogeneous population of X208 plaques. 2. X208 phages mixed with the cDNA library. 3. Homogeneous population of X200 plaques. 4. X200 phages mixed with the cDNA library.

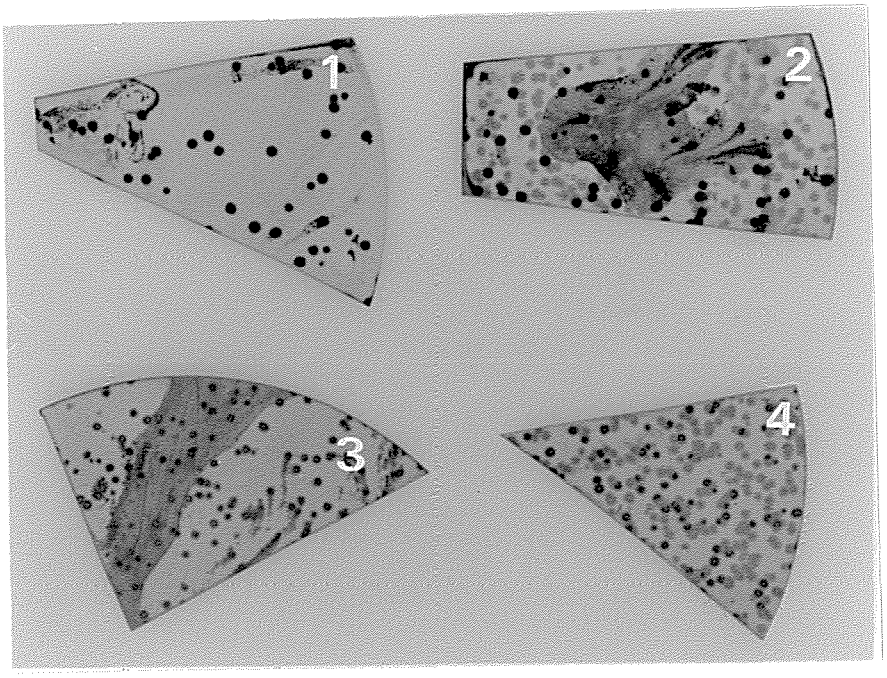

1
2
3
4

Fig. 6-7 *Eco*R1 digestion of recombinant phages X208 and X200 DNAs. Lane 1. X200 phage DNA. Lane 2. X208 phage DNA.

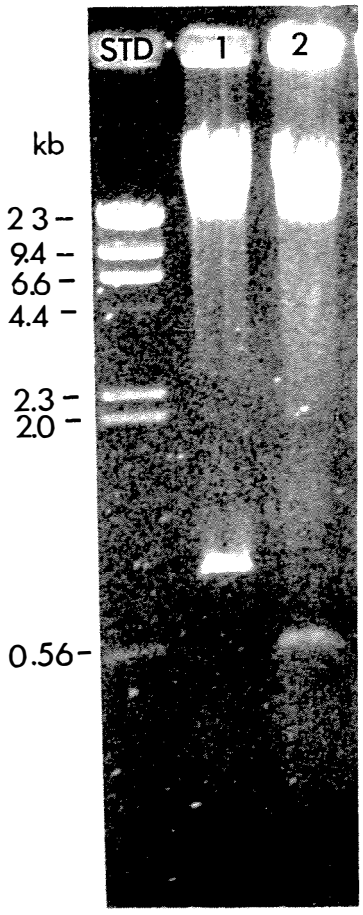

STD
1
2
kb
23 –
9.4 –
6.6 –
4.4 –
2.3 –
2.0 –
0.56 –

Fig. 6-8 A. *Exo*III digestion of a cDNA insert of the recombinant phage X200 B. *Eco*R1 and *Hind*III digestion of pGEM 4Z plasmids with the shortened cDNA inserts.

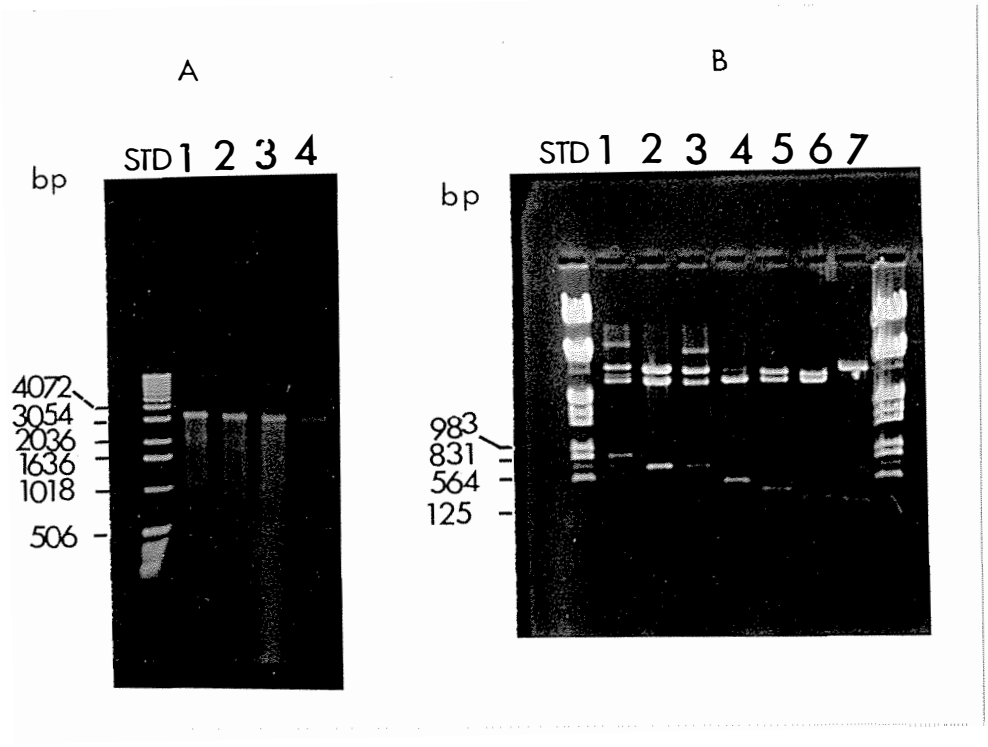

A
STD 1 2 3 4
bp
4072
3054
2036
1636
1018
506
B
STD 1 2 3 4 5 6 7
bp
983
831
564
125

Fig.6-9 Nucleotide sequence of the cDNA insert of the recombinant phage X200 and its comparison with that of a bacterial gene *ilv*C encoding acetohydroxy acid isomeroreductase.

```
                                                10        20        30
X200                                          GAATTCGCCGACGGCGCGAGCTACCTTCAG
                                              !!!!!!!!!! !!!!!!!!!!!!!!!!!!
Ecollv CTGGGCAAATGTCGCTTTATGGGCCGCGATGAATTCGCCGATGGCGCGAGCTACCTTCAG
      1200      1210      1220      1230      1240      1250

              40        50        60        70        80        90
X200   GGTAAAAAAGTGGTTATCGTCGGCTGTGGCGCACAGGGGCTGAACCAGGGCCTGAACATG
       !!!!!!!!!! !! !!!!!!!!!!!!!!!!!!!!!!!! !!!!!!!!!!!!!!!!!!!!
Ecollv GGTAAAAAAGTAGTCATCGTCGGCTGTGGCGCACAGGGTCTGAACCAGGGCCTGAACATG
      1260      1270      1280      1290      1300      1310

             100       110       120       130       140       150
X200   CGTGATTCTGGTCTCGATATCTCCTACGCTCTGCGTAAAGAAGCGATTGCCGAGAAGCGC
       !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
Ecollv CGTGATTCTGGTCTCGATATCTCCTACGCTCTGCGTAAAGAAGCGATTGCCGAGAAGCGC
      1320      1330      1340      1350      1360      1370

             160       170       180       190       200       210
X200   GCGTCCTGGCGTAAAGCGACCGAAAACGGTTTTAAAGTTGGTACTTACGAAGAACTGAT-
       !!!!!!!!!!!!!!!!!!!!!!!!!! !!!!!!!!!! !!!!!!!!!!!!!!!!!!!
Ecollv GCGTCCTGGCGTAAAGCGACCGAAAATGGTTTTAAAGTGGGTACTTACGAAGAACTGATC
      1380      1390      1400      1410      1420      1430

              220       230       240       250       260       270
X200   CCGCAGGCGGATCTGGTGGTTAACCTGACGCCGGACAAGCAGCACTCTGATGTAGTGCGC
       !! !!!!!!!!!!!!!! !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
Ecollv CCACAGGCGGATCTGGTGATTAACCTGACGCCGGACAAGCAGCACTCTGATGTAGTGCGC
      1440      1450      1460      1470      1480      1490

              280       290       300       310       320       330
X200   ACCGTACAGCCACTGATGAAAGACGGCGCGGCGCTGGGTTACTCGCACGGTTTCAACATC
       !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!! !!!!!!!!!!!!!!!!!!!!
Ecollv ACCGTACAGCCACTGATGAAAGACGGCGCGGCGCTGGGCTACTCGCACGGTTTCAACATC
      1500      1510      1520      1530      1540      1550

              340       350       360       370       380       390
X200   GTCGAAGTGGGCGAGCAGATCCGTAAAGATATCACCGTAGTCATGGTTGCGCCGAAATGC
       !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!! !!!!!!!!!!!!!!!!
Ecollv GTCGAAGTGGGCGAGCAGATCCGTAAAGATATCACCGTAGTGATGGTTGCGCCGAAATGC
      1560      1570      1580      1590      1600      1610

              400       410       420       430       440       450
X200   CCTGGCACCGAAGTGCGTGAAGAGTACAAAGGTGGATTCGGCGTACCGACGCTGATTGCC
       !! !!!!!!!!!!!!!!!!!!!!!!!!!!!! !!!! !!!!!!!!!!!!!!!!!!!!!!!!
Ecollv CCAGGCACCGAAGTGCGTGAAGAGTACAAACGTGGGTTCGGCGTACCGACGCTGATTGCC
      1620      1630      1640      1650      1660      1670

              460       470       480       490       500       510
X200   GTTCACCCGGAAAACGATCCGAAAGGCGAAGGTATGGCGATCGCTAAAGCATGGGCGGCA
       !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!! !!!!!!!! !! !!!!! !!!!!!!!
Ecollv GTTCACCCGGAAAACGATCCGAAAGGCGAAGGCATGGCGATTGCCAAAGCCTGGGCGGCT
      1680      1690      1700      1710      1720      1730
```

(to be continued)

Fig. 6-9 (...continued)

```
             520       530       540       550       560       570
X200   GCAACCGGTGGTCACCGTGCGGGCGTGCTGGAATCGTCCTTCGTTGCGGAAGTGAAATCT
       !!!!!!!!!!!!!!!!!!!!!!! !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
Ecollv GCAACCGGTGGTCACCGTGCGGGTGTGCTGGAATCGTCCTTCGTTGCGGAAGTGAAATCT
      1740      1750      1760      1770      1780      1790

             580       590       600       610       620
X200   GACCTGATGGGTGAGCAAACCATCCTGTGCGGTATGTTGCA-GCTGGCTCGCTGCTGTGC
       !!!!!!!!!!! !!!!!!!!!!!!!!!!!!!!!!!!!!! !!!!!!!! !!!!!!!!!
Ecollv GACCTGATGGGCGAGCAAACCATCCTGTGCGGTATGTTGCAGGCTGGCTCTCTGCTGTGC
      1800      1810      1820      1830      1840      1850

     630       640       650       660       670       680
X200   CTTCCGACAAGCTGGTGGAAGAGGGCACCGATCCGGCATACGCAGAAAAACTAATTCAGT
         ! !!!!!!!!!!!!!!!!!! !! !!!!!!!! !!!!!!!!  !!!!!!! !!!!!!!
Ecollv --TTCGACAAGCTGGTGGAAGAAGGTACCGATCCAGCATACGCGAAAAAACTGATTCAGT
        1860      1870      1880      1890      1900      1910

     690       700       710       720       730       740
X200   TCGGTTGGGAAACCATCACCGAAGCGCTGAAGCAGGGCGGCATCACCCTGATGATGGACC
       !!!!!!!!!!!!!!!!!!!!!!!!! !!!!! !!!!!!!!!!!!!!!!!!!!!!!!!!!
Ecollv TCGGTTGGGAAACCATCACCGAAGCACTGAAACAGGGCGGCATCACCCTGATGATGGACC
        1920      1930      1940      1950      1960      1970

     750       760       770       780       790       800
X200   GTCTCTCTAACCCGGCGAAACTGCGTGCTTACGCGCTGTCTGAACAGCTGAAAGAGATCA
       !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!! !!!!! !!!!!!!!!!!!!!!!!!!!!
Ecollv GTCTCTCTAACCCGGCGAAACTGCGTGCTTATGCGCTTTCTGAACAGCTGAAAGAGATCA
        1980      1990      2000      2010      2020      2030

     810       820       830       840       850       860
X200   TGGCACCCCTGTTCCAGAAACATATGGACGACATCATCTCCAGCGAATTC
       !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!! !!!!!!!!
Ecollv TGGCACCCCTGTTCCAGAAACATATGGACGACATCATCTCCGGCGAATTCTCTTCCGGTA
        2040      2050      2060      2070      2080      2090

Ecollv TGATGGCGGACTGGGCCAACGATGATAAGAAACTGCTGACCTGGCGTGAAGAGACCGGCA
        2100      2110      2120      2130      2140      2150
```

Eco R1

```
  1  GAATTCCGGA TCCATAATCC GCTGGCTATG CACCAGATTT TGCCAGCTCA CGCCATAAGG
       E  F  R  I  H  N  P  L  A  M  H  Q  I  L  P  A  H  A  I  R

 61  ATTGCCGACG ATATCTGCCA CCTTACCCAC CAGCACGGTT GGCACGCCTG CTTCATACAG
       I  A  D  D  I  C  H  L  T  H  Q  H  G  W  H  A  C  F  I  Q

121  TTTTTGTGGC ACCTGCACTT TTTCATCGAC GCCATAGCCC ATATGCACGA CCTGGAAACC
       F  L  W  H  L  H  F  F  I  D  A  I  A  H  M  H  D  L  E  T

181  GTTGTCATAA GCGCCAGAAC GCGGCGCATT GATACCAATA AAGCGCCCTT CTTTGCTTTC
       V  V  I  S  A  R  T  R  R  I  D  T  N  K  A  P  F  F  A  F

241  TGCGGCATCG AGAATGCGTT GACTGTCGGT TAACAGGCCA CCAAATGTAA TGACCCGACC
       C  G  I  E  N  A  L  T  V  G  =

301  GACCGGTACC TGCTCTCGCA CGATACGACC AATTTTGATT GCGTCGTCAA AAGAGATCAC

361  AGAGAGATTG GCGGTAATGT TATAGACCTG GCCTAAATCC GCCTCGAGAT TATCGCCAAT

421  CGCAACCGCC TGATTGACCC ACAGAAATTG CAGTTCATCG CCACGGNGCC GGAATTC
                                                          Eco R1
```

Eco R1

```
  1  GAATTCCGGC ACCGTGGCGA TGAACTGCAA TTTCTGTGGG TCAATCAGGC GGTTGCGATT
       E  F  R  H  R  G  D  E  L  Q  F  L  W  V  N  Q  A  V  A  I

 61  GGCGATAATC TCGAGGCGGA TTTAGGCCAG GTCTATAACA TTACCGCCAA TCTCTCTGTG
       G  D  N  L  E  A  D  L  G  Q  V  Y  N  I  T  A  N  L  S  V

121  ATCTCTTTTG ACGACGCAAT CAAAATTGGT CGTATCGTGC GTGAGCAGGT ACAGGTCGGT
       I  S  F  D  D  A  I  K  I  G  R  I  V  R  E  Q  V  Q  V  G

181  CGGGTCATTA CATTTGGTGG CCTGTTAACC GACAGTCAAC GCATTCTCGA TGCCGCAGAA
       R  V  I  T  F  G  G  L  L  T  D  S  Q  R  I  L  D  A  A  E

241  AGCAAAGAAG GGCGCTTTAT TGGTATCAAT GCGCCGCGTT CTGGCGCTTA TGACAACGGT
       S  K  E  G  R  F  I  G  I  N  A  P  R  S  G  A  Y  D  N  G

301  TTCCAGGTCG TGCATATGGG CTATGGCGTC GATGAAAAAG TGCAGGTGCC ACAAAAACTG
       F  Q  V  V  H  M  G  Y  G  V  D  E  K  V  Q  V  P  Q  K  L

361  TATGAAGCAG GCGTGCCAAC CGTGCTGGTG GGTAAGGTGG CAGATATCGT CAACAATCCT
       Y  E  A  G  V  P  T  V  L  V  G  K  V  A  D  I  V  N  N  P

421  TATGGCGTGA GCTGGCAAAA TCTGGTGGAT AGCCAGCGGA TTATGGATCC GGAATTC
       Y  G  V  S  W  Q  N  L  V  D  S  Q  R  I  M  D  P  E  F
                                                          Eco R1
```

signals or poly(A^+) tails which most mRNAs have. This might be due to incomplete synthesis of the second strand of cDNA, which could result in cleavage of any overhangs of the first strand cDNAs, thus leading to the loss of these sequences of the two clones. Alternatively, the possible presence of internal *Eco*R1 sites in these two cDNAs could also lead to the loss of these sequences during the subcloning steps.

Sequence analysis showed that the cDNA insert of the X200 clone was 96% identical with a bacterial gene *ilv*C which encodes acetohydroxy acid isomeroreductase, the second enzyme in the parallel isoleucine-valine biosynthetic pathway (Fig.6-9). Amino acid composition of this bacterial enzyme is different from that of CBL1 (Wek and Hatfield, 1986). It was not demonstrated that sequences of putative peptides of the X200 cDNA were homologous to those of the peptides of CBL1. Furthermore, nucleotide sequences based on backtranslation of CBL1 peptide sequences have also shown no good homology with the X200 DNA sequence. Thus, X200 cDNA does not encode CBL1. This conclusion correlates with the lower intensity of their immunoscreening blot (Fig. 6-6). However, the *Lac*Z fusion protein encoded by the X200 cDNA might share similar epitopes to CBL1, which resulted in a positive reaction with anti-CBL1 serum, or the *Lac*Z fusion protein of X200 cDNA might be recognized by other antibody molecules which are present in anti-CBL1 serum, but which are not specific to CBL1.

The cDNA insert of X208 clone did not have good homology with any sequences in the data bases, thus, it was a new sequence. However, the putative peptide sequences (Fig.6-10) encoded by X208 cDNA also showed no apparent homology with those of CBL1 peptides. In addition, the sequence of the cDNA insert of the X208 clone showed no significant homology with DNA sequences based on backtranslation of the peptide sequences. The amino acid composition of the two putative peptides encoded by the

X208 cDNA insert (Table 6-2) did not show good homology with that of CBL1. However, it could not be ruled out that the X208 cDNA could encode one domain of CBL1, and peptides which were sequenced could be confined to another domain. If the X208 cDNA encoded part of CBL1, the peptide would resemble part of the newly synthesized CBL1 in tamarillo. Amino acid composition of nascent CBL1 would be different to that of mature CBL1 which could have undergone post-translational modification. WGA, a chitin binding lectin from wheat germ, undergoes cleavages of N-terminal and carboxyl terminal sequences of nascent WGA (pre-pro form) during the extensive post-translational modification process (Mansfield *et al.*, 1988).

Table 6-2 Amino acid composition of CBL1 and two putative peptides (pep1 and pep2) encoded by the X208 cDNA insert, which were expressed in bacteria.

AA residues	CBL1	pep1	pep2	AA residues	CBL1	pep1	pep2
Hyp	6.5	-	-	Met	0.9	2	2
Asx	12.9	8	21	Ile	1.0	11	12
Thr	7.0	5	4	Leu	4.8	7	11
Ser	19.8	1	7	Tyr	6.5	0	5
Glx	23.3	6	20	Phe	3.0	8	7
Pro	16.0	3	5	His	1.3	11	2
Gly	25.8	3	16	Lys	10.2	1	5
Ala	7.7	11	11	Arg	5.0	5	9
Cys	28.8	3	-	Trp	ND	2	2
Val	2.6	3	20				

However, it still possible that the X208 cDNA might not encode CBL1 but encodes a protein closely related to CBL1, which could be recognized by the anti-CBL1 serum. That this protein could not be detected in the western blot might be due to the very small quantity of this protein which is present in tamarillo fruits.

To confirm whether X208 cDNA did in fact encode CBL1, future work on this line would be the sequencing of more peptides to identify any homology with the sequence of the X208 cDNA insert. Once it is confirmed, the X208 cDNA could be used as a probe to isolate a full size CBL1 cDNA. Future work could also be directed on further characterization of other positive cDNAs obtained from this work.

In terms of improvement in the immunological screening of the cDNA library, one could use a purified antibody specific to CBL1. Although the anti-CBL1 serum used in this study was specific as indicated by western blot data, highly purified CBL1-specific antibody might have a better chance to reduce the possibility of false positives. CBL1-specific antibody could be readily purified on a CBL1 coupled column, e.g. CBL1 could be immobilized on cyanogen-bromide-activated Sepharose 4B (Harlow and Lane, 1988).

According to the criteria set by Sambrook *et al.*, (1989), the specificity and sensitivity of anti-CBL1 serum should be sufficient for immunoscreening for CBL1, unless the *Lac*Z fused CBL1 was unstable. Since no obvious clone encoding CBL1 was isolated, the fusion CBL1 (F-CBL1) might be very labile or toxic to the *E. coli* host. In addition, anti-CBL1 serum might not show the same specificity and sensitivity towards F-CBL1 expressed in bacteria as towards CBL1 purified from tamarillo due to structural differences between F-CBL1 expressed in bacteria and CBL1 of plant origin: 1. F-CBL1 may not contain hydroxyproline residues and carbohydrate moieties since bacteria may not have the same post-translational modification systems as tamarillo; 2. F-CBL1 is likely to have different disulphide isomerizations, as bacteria may have a different protein disulphide isomerase; 3. F-CBL1 may not have the same peptide backbone around the planar imide bonds in the sequence -X-Pro- , i.e. *cis* or *trans* conformers,

since bacteria may not have the same prolyl-peptidyl *cis/trans* isomerase (PPI) as tamarillo. Further more, F-CBL1 would remain with the β-galactosidase. Therefore, the extensive structural changes may occur and render it difficult for anti-CBL1 IgG to recognize F-CBL1 with a very high sensitivity, which might be the cause of unsuccessful isolation of CBL1 cDNA clone.

Thus, future work could be focused on a cDNA library constructed on λgt10, which will be screened with an oligonucleotide probe synthesized according to the T12 peptide sequence. This has been synthesized during this study. cDNA inserts in λgt10 vector will not be expressed, thus there will be no possible problems encountered in the immunoscreening. In addition, the clone encoded CBL1 could be screened with 6 times higher possibility than that cloned in λgt11. In the latter case, only one of six CBL1 clones have the chance to be expressed in-frame.

6.3 Summary

The N-terminal sequence and sequences of 4 tryptic peptides of CBL1 were determined. The amino acid compositions of these peptide sequences were in general agreement with that of CBL1. CBL1 could be evolutionarily related to the Gramineae lectins since they have similar chemical compositions and sugar specificities. CBL1 tryptic peptide sequences showed homology with the Gramineae lectins although based on limited sequence inforation of CBL1.

Attempts to isolate CBL1 cDNA clones were made. Two clones showing positive reaction with anti-CBL1 serum were sequenced. The sequence of the first cDNA clone (X200) showing a weak positive reaction with the anti-CBL1 serum was

determined. It did not appear to encode for CBL1. A database search showed that the X200 cDNA clone is 96 % identical with a bacterial gene *ilv*C which encodes acetohydroxy acid isomeroreductase. The sequence of the second cDNA clone (X208) showed a stronger reaction with the anti-CBL1 serum but with no apparent homology to CBL1 peptide sequences and nucleotide sequences based on backtranslation of these CBL1 peptide sequences. The X208 clone could encode one domain of CBL1, and peptides which were sequenced could be confined to another domain.

The complexity of the immunoscreening method for this unusual extracellular lectin is discussed. Although a complete CBL1 cDNA clone was not isolated, this study provided tryptic peptide sequences of CBL1, which would be useful for future work on the analysis of the primary sequence of this interesting lectin from tamarillo fruit.

CHAPTER 7 GENERAL CONCLUSION AND SUMMARY

Several *Cyphomandra* species were found to contain high agglutination activity specific to oligomers of N-acetylglucosamine. Thus, *Cyphomandra* species can be considered as a new source of lectins.

New lectins (designated as CBL1 and CBL2) from *Cyphomandra betacea* (red tamarillo) were identified and purified. Attempts to undertake biochemical characterization, subcellular localization and molecular sequence analysis were made. CBL2, which was immunologically unrelated to CBL1, was not further characterized.

CBL1 was an abundant protein in fruit extracts, and was predominant in the fruit juice. CBL1 could be readily purified to homogeneity using affinity and ion exchange chromatography. Gel filtration analysis of CBL1 showed that it was approximately 50 kDa, and SDS-PAGE analysis with or without β-mercaptoethanol treatment showed that it gave a single band with molecular size of 25 kDa. It was thus concluded that CBL1 is composed of two identical monomers linked by non-covalent interactions. N,N',N",N""-tetraacetylchitotetraose was the best carbohydrate inhibitor of CBL1-induced agglutination of rabbit erythrocytes.

CBL1 consists of abundant residues of Cys (16 %), Gly (14 %), Glx (13 %), Ser (11 %), Pro (9 %) and Asx (7 %), and to a lesser extent, hydroxyproline residues. These characteristics are similar to other solanaceous lectins. However, CBL1 has its own unique structural properties. CBL1 showed much more stability than any other solanaceous lectins reported to date, suggesting many intra-peptidal disulphide bridges might

exist since Cys residues accounted for 16 % of total amino acid residues.

Despite the general resemblance of CBL1 to other solanaceous lectins in terms of chemical composition and carbohydrate specificities, cross-reactions among solanaceous lectins in double immunodiffusion tests using anti-LEL and anti-CBL1 sera have not been demonstrated, suggesting they may have different epitope structures.

Immunocytological study revealed that CBL1 predominantly associated with the cell walls of fruit tissue, suggesting that CBL1 was fruit tissue specific and might play a defence or communication role for seed development.

Although CBL1 was found to be a glycoprotein and contained small amount of hydroxyproline residues, WGA and other Gramineae lectins showed similar amino acid composition and sugar specificities to those of CBL1. Furthermore, sequences of tryptic peptides of CBL1 showed some homology with the Gramineae lectins such as WGA. Therefore, CBL1 could be evolutionarily related to the Gramineae lectins.

Two cDNA clones which were reactive to anti-CBL1 serum were sequenced. One cDNA clone (X200) showing a weak reaction with anti-CBL1 serum was found to be 96 % identical with a bacterial gene *ilvC* encoding acetohydroxy acid isomeroreductase. It did not encode CBL1. Another cDNA clone (X208) showing a much stronger reaction with anti-CBL1 serum was a new sequence, however, putative peptide sequences of this clone did not match the sequences of CBL1 peptides, nor did nucleotide sequences back-translated from these CBL1 peptides match the cDNA sequence of X208. Thus, X208 cDNA

clone could encode one domain of CBL1 or a closely related protein, while the tryptic peptides of CBL1 could be confined to another domain of CBL1.

This thesis has studied a new lectin from *Cyphomandra betacea*, and has opened up a new area of research on solanaceous lectins from the genus *Cyphomandra*. Further study on this group of lectins at the molecular level could be directed at their gene structures, expression and regulations.

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APPENDIX CLASSIFICATION OF PLANT LECTINS (Wu *et al.*, 1986)

I. N-Acetylglucosamine Specific Lectins

I-1. Chitin oligosaccharide specific lectins

APL-I, *Aaptos papillata* lectin
APL-II & III, *Aaptos papillata* II & III lectins
CPL, *Cucurbita pepo* lectin
CML, *Cucurbita maxima* lectin (Pumpkin)
CML-II, *Cytisus multiflorus*-II lectin
CSL, *Cytisus sessilifolius* lectin
DSL, *Datura stramonium* lectin
GSL-II, *Griffonia (Bandeiraea) simplicifolia* II lectin
LEL, *Lycopersicon esculentum* lectin (Tomato)
LAL, *Luffa acutangula* lectin (Ridge Gourd)
FAL, *Phytolacca americana* Pa-2 lectin (Pokeweed)
STL, *Solanum tuberosum* lectin (Potato)
UDL, *Urtica dioica* lectin (Stinging nettle)
WGA, *Triticum vulgaris* agglutinin (Wheat germ)
UEL, *Ulex europaeus* II lectin (See $_L$Fuc specific lectins)

I-2. Other GlcNAc-specific lectins requiring further characterization

LAL, *Laburnum alpinum* lectin
BSL, *Brachypodium sylvaticum*
DLL, *Dolichos lablab* lectin
HVL, *Hordeum vulgare* lectin (Barley)
Rice lectin, *Oryza sativa* lectin
SCL, *Secale cereale* lectin (Rye)

II. N-Acetylgalatosamine specific lectins

II-1. GalNAcα1,3GalNAc specific lectins

DBL, *Dolichos biflorus* lectin (Horse Gram)
ABrL, *Amphicarpaea bracteata* lectin (Hog-peanut)
WFL, *Wisteria floribunda* lectin

II-2. GalNAcα1,3Gal (Human blood group A) specific lectins

GSI-A_4, *Griffonia simplicifolia* A_4
LBL, *Phaseolus lunatus* lectin (Lima bean)
SBL, *Glycine max* lectin (Soy bean)
VVL, *Vicia villosa* lectin

II-3. GalNAcα1→Ser ar Thr (Tn) specific lectin

VVL-B_4, *Vicia villosa* B_4 lectin
SSl, *Salvia sclarea* lectin
MPL, *Maclura pomifera* lectin
BPL, *Bauhinia purpurea alba* lectin

II-4. Other GalNAc specific lectins requiring further Characterization

APL, *Aegopodium podagraria* lectin
BDL, *Bryonia dioica* lectin
CAL, *Caragana arborescens* lectin (Pea tree)
CTL, *Clerodendron trichotomun* lectin
CNL, *Clitocyl nebularis* lectin
DDL, *Dictyostelium discoideum* I lectin (Discoidin I)
EHyL, *Eranthis hyemalis* lectin
EHeL, *Euphorbia heterophylla* lectin
FFL, *Fomes fomentarius* lectin
HCL, *Hura crepitans* seed lectin
MAL, *Macrotyloma axillare* anti-A lectin
MCL, *Momordica charatia* lectin (Bitter pear melon)
OHL, *Ononis hircina Jacq* lectin
PTL, *Psophocarpus tetregonolobus* lectin (Wing bean)
RPL, *Robinia pseudacacia* lectin
TML, *Tridacna maxima* lectin
VCL, *Vicia cracca* anti-A lectin
VAL-II & III, *Viscum album*-II & III lectins

III. Mannose and/or Glucosé Specific Lectins

III-1. Manl-linked oligosaccharide-specific lectins

ConA, *Concanavalia ensiformis* agglutinin (Jack Bean)
LCL, *Lens culinaris* lectin
PSL, *Pisum sativum* lectin (Pea)
VFL, *Vicia faba* lectin (Fava bean)

III-2. Other Man and/or Glc-specific lectins requiring further characterization

DGL, *Dioclea grandiflora* lectin
LOL, *Lathyrus odoratus* lectin (Sweet pea)
LSL, *Lathyrus sativum* lectin (Chickling pea)
LTL, *Lathyrus tingitanus* lectin (Tangier pea)
OVL, *Onobrychis vicifolia* lectin (Sainfoin)
PFL, *Perca fluriatilis* lectin
POL, *Ptilotis obovatus* lectin
RCL, *Rhadogia cassifolia* lectin
VCL, *Vicia cracca* lectin (Vommon vetch)
VSL, *Vicia sativa* lectin

IV. Galactose specific lectins

IV-1. Galβ1,3GalNAc(T) specific lectins

PNA, *Arachis hypogaea* lectin
BPL, *Bauhinia alba* lectin
MPL, *Maclura pomifera* lectin (Osage orange tree)
SJL, *Sophora japonica* lectin (Jap. pagoda tree)
RCA^2, Ricin *Ricinus communis*
VGL, *Vicia graminea* lectin (Blood group **N** specific)
ABil, *Agaricus bisporus* lectin (Mushroom)
ARL, *Agropyrum repens* lectin (Couch grass)
AIL, *Artocarpus integrifolia* lectin (Jacalin)
ALL, *Artocarpus lakoocha* lectin

IV-2. Galβ1,3(4)GlcNAc (Human blood group I and II carbohydrates sequences) specific lectins

RCA_1, *Ricinus communis* agglutinin (Castor bean)
TAL, *Datura stramonium* lectin (Datura,Thorn apple)
ECL, *Erythrina cristagalli* lectin (Coral tree)
GCL, *Geodia cydonium* lectin
Other *Erythrina* lectins
E.caffra[+] ,*E.corallodendron, E. flabelliformis, E. humeana, E. latissima, E.lysistemon, E. perrieri*[+], *E. stricta, E. zeyheri.*
PHA-E, *Phaseolus vulgaris* isolectin E^4 (Red kidney bean)
PAL, *Phytolacca americana* lectin (pokeweed mitogen)
PHA-L, *Phaseolus vulgaris* isolectin L^4 (Leukoagglutinin)
EHL, *Eranthis hyemalis* agglutinin (from Winter-aconite root rubers)

PNL, *Arachis hypogaea* lectin (See Galβ1,3GalNAc specific lectins)
BPL, *Bauhinia purpurea alba* lectin (See Galβ1,3GalNAc specific lectins)
Ricin (See Galβ1,3GalNAc specific lectins)
UDL, *Urtica dioica* agglutinin (Stinging nettle)
SNL, *Sambucus nigra* lectin

IV-3. Galα1,3Gal (B) specific lectin

GSL-B_4, *Griffonia simplicifolia* I-B_4 lectin

IV-4. Other Gal-specific lectins and those required for further classification

AXP-I and II, *Axinella polypoides* I and II lectins
APL, *Abrus precatorius* lectin (Jequirity bean)
AML, *Ascidia malaca* lectin
ABL, *Abramis brama* lectin
BCL, *Bauchinia carronii* lectin
BFL, *Butea fronosa* lectin
CJL, *Crotalaria juncea* lectin
CML-1, *Cytus multiflorus* lectin
CSL, *Cytisus scoparius* lectin
DCL, *Didemnum candidum* lectin
DDL-II, *Dictyostelium discoideum* lectin
EAL, *Erythinia arborescens* lectin
EIL, *Erythinia indica* lectin
ELL, *Erythinia lithosperma* lectin
ESL, *Erythrina suberosa* lectin
ECL, *Euphorbia characias* lectin
HCL, *Hardenbergia comtoniana* lectin
HRL, *Halocynthia riretzi* lectin
HCL, *Hura crepitans* lectin
LAL, *Laccaria amythstina* lectin
OVL, *Octopus vulgaris* lectin
PAL, *Phaseolus aureas* lectin (Mung bean)
RRL, *Rutilus rutilus* lectin
SSL, *Sarothamnus scoparius* lectin
SEL, *Scardinus erythrophtalmus* lectin
VVL, *Vimba vimba* lectin
TKL, *Trichosanthes kirilowii* lectin
VAL-I, *Viscom album* I lectin
VUL, *Vigna unguiculata* lectin (Cowpeas)

V. $_L$Fucose Specific Lectins

V-1. Monofucosyl specific lectins

CSL, *Cytisus sessilifolius* lectin
EEL, *Euonymus europeus* lectin (Spindle tree)
LAL, *Laburnum alpinum* lectin
LTL, *Lotus tetragonolobus* lectin (Asparagus pea)
UEL-I *Ulex europaeus*-I lectin (Gorse seed)
UEL-II, *Ulex europaeus*-I lectin (Gorse seed)

V-2. Difucosyl specific lectins

GSL-IV, *Griffonia simplicifolia* IV lectin

V-3. Other $_L$Fuc-specific lectins required further characterization

LAF, *Laccaria amethystina* lectin

VI. Sialic Acid Specific Lectins

Avocado lectin,
SML, *Sambucus nigra*
WGA, *Triticum vulgaris* agglutinin (See Section **I-1**)
LSL, *Lactuca scariole* lectin

CORRECTIONS:

The method for determining mitogenic activity (p.44) should be amended to include a positive control. Phytohaemagglutinin (PHA) (0.1 ml of 10 mg/ml reconstituted PHA) was incorporated into the assay and stimulated an increased frequency of mitotic figures.

In Fig. 4-1 (p. 94), no precipitin line was found between wells 5 and 6 in the top panel of A (A,N). Yet the same wells in A, T produced a massive response. The reason for this discrepency is unknown, but could be due to antigen concentration.

DNA sequencing has been repeated twice (p.74). One strand of X208 was sequenced, and both strands of most X200 were sequenced. Sequence data was manually input for sequence analysis and the accuracy was checked twice (p. 74).

The homology (p. 121, 122) between X200 and the bacterial gene encoding acetohydroxy acid isomeroreductase needs further discussion. X200 DNA might be the result of bacterial DNA contamination during cDNA library construction since it is unlikely that a tamarillo DNA has 96% homology to a bacterial DNA fragment. The source of the possible contamination is not known. And the extent of the possible bacterial DNA contamination is not clear. The isolation of the X200 clone by the immunoscreening method described in the thesis indicates it encodes a peptide which has some epitopes recognized by anti-CBL1 serum.

A potential polyadenylation signal site was not found for X200 and X208 clones (p.121-123). Also an obviuous clone for encoding CBL1 was not found. Although one could study other positive clones isolated during this study, the present library may be not of high quality. It is prudent that a new cDNA library constructed on lambda gt 10 vector is needed in order to isolate a cDNA clone encoding CBL1 (as discussed on p.127).

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