DISCUSSION

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The occurrence of more than one lectin with different sugar specificity in seeds of leguminous plant is a common phenomenon. For instance, lectins specific to fucose and GlcNAc have been isolated from the seeds of *Ulex europaeus* (Matsumoto *et al.*, 1969). Konami *et al.* (1983 and 1991) also reported that two kinds of lectin inhibited by GlcNAc and galactose/lactose have been isolated from both the seeds of *Laburrum alpinum* and *Cystisus sessilifolius*. In addition, Mo *et al.* (1990) have found a new lectin specific to galactose/lactose other than the mannose-reactive lectin in seeds of *Dolichos lablab*. The presence of a mannose-specific lectin other than the galactose specific one in the seeds of *Vicia villosa* (Qian *et al.*, 1994) and *Artocarpus heterophyllus* (Misquith *et al.*, 1994) have further furnished the list.

The data from our laboratory have previously established the presence of a galactose- and IgA1-binding lectin, termed lectin C, in seeds of champedak (*Artocarpus integer,* Hashim *et al.,* 1991 and 1993). The present study was directed at the isolation and characterisation of a second lectin with distinctive reactivity from the same seed extract. This mannose-binding lectin, termed lectin-M, was markedly different from lectin-C in terms of its physico-chemical properties, carbohydrate specificity and mitogenicity.

4.1 ISOLATION AND STRUCTURAL CHARACTERISATION OF CHAMPEDAK LECTIN M

The mannose-binding lectin was isolated from the effluent of immobilised galactose affinity chromatography. The effluent was subjected to immobilised mannose affinity chromatography and the bound fractions were eluted using 0.8 M

mannose in PBS. When a comparative experiment was performed with crude seed extract of jackfruit (*Artocarpus heterophyllus*), it was apparent that substantially more mannose-reactive lectin may be obtained from the crude extracts of *Artocarpus integer* seeds than from the crude seed extracts of *Artocarpus heterophyllus*, with their respective yield of about 15 mg and 0.8 mg per gram of seeds. When compared to other lectins isolated from leguminous seeds such as pea, broad bean and lentil, this amount of mannose-binding lectin that could be obtained from *A. integer* seeds was approximately 15 fold greater. However, the recovery was two fold lesser as compared to the isolation of Con A from *Concanavalin ensiformis*.

Sephadex G-100 gel filtration chromatography performed on the mannose-binding preparation using 0.1 M acetic acid as the elution buffer generated only a single 64 kDa elution peak. The lectin was resolved into a single band with M_r of 16,800 when analysed by SDS-PAGE in the presence of a reducing agent. In its absence, however, two sharp bands with M_r of 16,800 and 31,400 were detected. The M_r obtained in the gel filtration and SDS-PAGE experiments suggest that the lectin existed mainly as tetramers of the polypeptide chain, with some of the polypeptides being disulphide-linked to give dimers. The structure of lectin-M from seeds of *Artocarpus integer* was thus similar but not quite identical with the artocarpin lectin (Misquith *et al.*, 1994) due to the presence of disulphide linkages.

4.2. INTERACTION OF LECTIN M WITH SELECTIVE GLYCO-PROTEINS

The functional property of lectin-M was assessed by observing their interactions with different isotypes of human immunoglobulins using simple double

diffusion and competitive HRP-binding assay. In the double diffusion experiment, it was observed that lectin-M interacted strongly with IgE and IgM but not with any other immunoglobulin isotypes. This interaction was confirmed when a competitive HRP binding assay was carried out in the presence of all the immunoglobulins. In this experiment, however, IgA2 was also noted to result in significant inhibition of the lectin-M-HRP interaction, although this was much weaker than that observed with IgE and IgM.

While all immunoglobulins contain oligosaccharide moieties attached to their selective asparagine residues, only IgE and IgM are known to contain the high-mannose type *N*-glycan in their structures (Baenziger *et al.*, 1974; Hickman *et al.*, 1972). Recent studies have however, revealed the presence of small amounts of high-mannose type *N*-glycan structures in human myeloma proteins of the IgA2 and IgA1 isotype (Endo *et al.*, 1994). It was therefore suspected that lectin-M may be selectively interacting with the mannose residues of the high mannose-type *N*-linked oligosaccharide moieties of glycoproteins. The reactivity studies of lectin-M were thus, extended to include several glycoproteins with established *N*-linked oligosaccharide structures.

When lectin-M was subjected to double diffusion experiments with other glycoproteins containing defined carbohydrate structures, the formation of precipitin lines were detected only with porcine thyroglobulin, human α_1-acid glycoprotein and horseradish peroxidase but not with human transferrin, human α_1-antitrypsin and ovalbumin. Among these glycoproteins, porcine thyroglobulin (Tsuji *et al.*, 1981), horseradish peroxidase (Kurosaka *et al.*, 1991) and ovalbumin (Yamashita *et al.*, 1978) have been reported to contain the high mannose-type *N*-glycan structures.

Human transferrin (Yamashita *et al.*, 1989), α_1-antitrypsin (Mega *et al.*, 1980) and α_1-acid glycoprotein (Schmid *et al.*, 1977) on the other hand, were known to be exclusively complex-type *N*-linked oligosaccharide-containing glycoproteins. Thus, the data from this experiment were not in support of our initial hypothesis that the *Artocarpus integer* lectin-M had selectively interacted with high-mannose-type *N*-glycans of glycoproteins. This was especially clear when the competitive HRP-binding assay was carried out in the presence of the glycoproteins. All glycoproteins that were tested appeared to demonstrate some inhibitory effect, although differing in potency, on the lectin-M-HRP interaction.

4.3 EFFECT OF SUGARS AND OTHER SUBSTANCES ON HRP-LECTIN M INTERACTION

To assess the carbohydrate binding specificity of the champedak lectin-M, sugar inhibition studies were performed using a competitive HRP-binding assay. Among the monosaccharides, methyl-α-D-mannopyranose was the most potent inhibitor, followed by D-mannose which was marginally poorer. D-glucose was four times weaker as an inhibitor than D-mannose and the substitution of the C-2 hydroxyl group of D-glucose with an acetamido group as in *N*-acetyl-D-glucosamine had little adverse effect on the binding. These results suggest that the lectin could be classified as a mannose/glucose-binding lectin similar to Con A, artocarpin, pea lectin, and lentil lectin.

The fact that D-galactose and its derivatives showed no effect on the binding also confirmed that the mannose-binding lectin preparations were totally free from lectin-C. The heterogeneous structure and function of the two lectins from seeds of

Artocarpus integer was also confirmed by data of the double diffusion experiment where precipitin lines of non-identity were obtained.

Among the mannobioses, Manα1-3Man was the most potent inhibitor with the disaccharide exhibiting 8 times greater potency than D-mannose. The introduction of a methyl group at C-1 of the Manα1-3Man reduced its inhibitory potency greatly. Manα1-6Man was a weaker ligand than Manα1-3Man but still better than D-mannose. The introduction of a methyl group at C-1 of Manα1-6Man resulted in minimal changes to its inhibitory potency. Thus, Manα1-6Man1-*O*-Me was an inhibitor of similar potency as Manα1-6Man and Manα1-4Man1-*O*-Me. On the other hand, Manα1-2Man1-*O*-Me exhibited marginally poorer inhibitory potency compared to D-mannose. However, the substitution of Man with GlcNAc as in Methyl-6-*O*-(GlcNAc)-α-D-Man abrogated its inhibitory potency. Other mannose derivatives which did not show any inhibitory effect were *N*-acetyl-mannosamine, 6-deoxy-L-Man, D-Man-6-phosphate, D-Man-1-phosphate, 1,6 anhydro-β-D-Man and D-mannosamine.

From the inhibition studies, it generally appeared that the mannose-binding lectin-M from seeds of *Artocarpus integer* demonstrated a preference for Manα1-3Man as a ligand when compared to Manα1-6Man and Manα1-2Man. This is similar to artocarpin (Misquith *et al.,* 1994) and Snowdrop lectin (Shibuya *et al.,* 1988). For Con A, pea lectin and lentil lectin, Manα1-2Man is regarded as a better ligand (Kornfeld *et al.,* 1981; Brewer and Brown, 1979).

Similar competitive HRP-binding assays performed in the presence of EDTA also demonstrated that metal ions are not required for the lectin interaction. The observed inhibitory effect induced by EDTA occurred only at a high concentration,

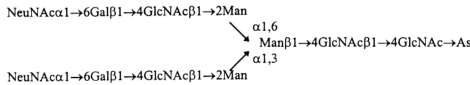
suggesting that it was a consequence of something other than the chelating effect. In this context, lectin M is consider to be rather unique because almost all known legume plant lectins required metal ions (Ca^{2+} and Mn^{2+}) for activity (Sharon, 1993).

4.4 BINDING SPECIFICITY OF LECTIN M-AFFINITY COLUMN

Various immobilised lectins have been successfully used for fractionation and for structural studies of glycoproteins (section 1.5.2). Lectin M as an entirely new man/glc-specific lectin may also provide an alternative choice in separation and structural analysis of glycoproteins.

The data of this study demonstrates that all the N-linked oligosaccharides containing glycoproteins tested were bound to the lectin M-affinity column regardless of the types of sugar chains they were bearing. For example, both orosomucoid (containing exclusively complex-type sugar chains) and ovalbumin (carry only high mannose- or hybrid-type sugar chains) showed a comparable significant binding to lectin M-Sepharose column. This observation is in total agreement with the finding in HRP-binding assay study where all high mannose-type and complex-type oligosaccharides containing glycoproteins tested exhibited inhibitory effect.

On the other hand, the complete retention of human transferrin, which predominantly (> 90 %) contained biantennary complex type sugar chain by lectin M-affinity column, suggests that lectin M demonstrated the strongest affinity towards the following biantennary complex-type oligosaccharide among the various other complex-type sugar chains:



Human α_1-antitrypsin which was partially retained by lectin M-affinity column also contained high percentage of this identical biantennary chain (Mega *et al.*, 1980). When desialylated, this biantennary chain is identical to a group of oligosaccharide present in human orosomucoid (Fournet *et al.*, 1978). With exception of ovalbumin, the other two glycoproteins tested i. e., human IgM and porcine thyroglobulin were also known to contain a certain amount of biantennary complex-type chains in addition to their high mannose-type oligosaccharides chains.

From the data, it can be concluded that lectin M preferentially binds to the biantennary complex-type N-linked oligosaccharide structures of glycoproteins. This is very similar to Con A from the seeds of *canavalia ensiformis* (Krusius *et al.*, 1976). The question of whether there exist structural differences between the N-linked oligosaccharides of fractions of glycoprotein that was eluted out of the column and the fraction of the same glycoprotein that was retained is certainly an interesting subject that warrants further investigation.

4.5 INTERACTION OF LECTIN M WITH HUMAN SERUM PROTEINS

With the exception of albumin and C-reactive protein, most serum proteins including immunoglobulin are glycosylated (Turner, 1992). The binding of plant lectins to these serum glycoproteins have been widely explored. (Nakamura *et al.*, 1960; Harris and Robson, 1963; Leon, 1967; and Goldstein and Hayes, 1978).

Preliminary study on interaction of lectin M with human serum protein using immunoelectrophoresis technique revealed that lectin M had interacted strongly with an unknown serum protein and weakly with some other serum proteins. To identify these lectin M-reactive human serum proteins, a high resolution two dimensional

electrophoresis was performed. The lectin M-reactive proteins were isolated from normal human serum using an immobilised lectin M column as described in the Materials and Methods (Section 2.5.6). Upon resolution by 2D electrophoresis and compared to a SWISS-2DPAGE reference (Sanchez *et al.*, 1995), the lectin M-reactive serum proteins were recognised as IgA, α_1-antitrypsin, and haptoglobin-β-chain.

In addition to these proteins, a few unidentified proteins were also detected. However, IgE, IgM, orosomucoid and transferrin, which have been shown to interact with lectin M in the HRP-binding assay were not detected in the bound fractions of lectin M-affinity column. The absence of these proteins in the lectin M-bound fractions was possibly due to their relative differences in affinity towards lectin M, and thus created competition for the limited binding site in the column. Alternatively the proteins may have been retained in the column and not able to be eluted by 0.1 M methyl-α-mannopyranose.

A more complete map of lectin M-reactive proteins could be obtained by using the enzyme-conjugated lectin M as a tool to probe the serum proteins which have been separated by 2D-electrophoresis and blotted on a nitrocellulose membrane. As visualised in the membrane (Fig. 17), enzyme-conjugated lectin M was able to pick up transferrin, IgM and orosomucoid, in addition to IgA, α_1-antitrypsin, and haptoglobin-β-chain which have been detected in bound fractions of lectin M-affinity chromatography 2D gel. The failure to detect IgE in this analysis was not quite unexpected as the concentration of this immunoglobulin is very low in normal human serum.

In addition to those detected serum proteins, γ-heavy chain of IgG which was not an inhibitor in the lectin M-HRP-binding assay, was found to be interactive. It is believed that this detection of γ-heavy chain in the lectin M-blotting experiment, was due to the exposure of carbohydrate chain as a result from the unfolding of IgG during the electrophoresis process prior to blotting. The inaccessibility of the N-linked oligosaccharide moieties of intact IgG molecules is quite an established phenomenon (Ryazantsev *et al.*, 1990).

4.6 MITOGENIC ACTIVITY OF LECTIN M

Earlier studies of our laboratory using crude seed extract of champedak revealed it as a potent mitogen of human PBMC (Hashim *et al.*, 1992). Since many lectins are mitogenic, it was initially suspected that the earlier galactose-reactive lectin C that was isolated from crude seed extract of champedak was responsible for the mitogenic activity shown by the crude extract. In the light of the discovery of a second lectin present in the champedak crude extract, a study was performed to readdress the mitogen component in the crude extract. Unfortunately, due to the difficulty to obtain a large amount of human lymphocytes, the mitogenic studies had to be performed on the murine system.

4.6.1 Effects on Proliferation of Murine T Cells

The dose responsive curve obtained from stimulatory study of murine T lymphocytes with crude extract, lectin M and lectin C clearly showed that the mitogenic activity shown by crude extract was attributed to lectin M. Lectin M stimulated a maximum response at a concentration of 2.5 μg/ml in a 3 days culture.

However, it revealed self inhibition at supraoptimal concentration, which is a common feature of all mitogenic lectins known so far (Niks *et al.*, 1990).

In the case of lectin C, a proliferative activity was also detected when a very high concentration of the lectin was used. This finding suggested that lectin C might be a different mitogenic lectin which possess poorer proliferative activity compared to lectin M. However, the possibility of contamination of lectin C in the preparation with lectin M can not be discounted. Further investigation is needed to clarify this situation.

The fact that addition of mannose resulted in the inhibition of the T-cell proliferation suggested that lectin M stimulates the cells by binding to carbohydrate-containing reactive sites. Nevertheless, a complete inhibition was not obtained even in the presence of 5 mg/ml mannose. This is perhaps because mannose is not potent enough to compete with cell sites for the carbohydrate binding site of lectin. On the other hand, it is also possible that the time relationship for DNA synthesis are altered in the presence of mannose. Maximal rates of DNA synthesis occurring at time other than 72 hr would not be revealed by these experiments.

4.6.2 Effect on Proliferation of Murine B Cells

Spleens of congenitally athymic nude mice have been shown to be deficient in T cells (Raff and Wortis,1970). Thus, the absence of proliferation when nude mouse spleen cells were subjected to lectin M stimulation proved that lectin M is not a B cell mitogen. This finding is in total agreement with the previous observation in which crude extract has been shown to selectively stimulate human T cells (Hashim *et al.*, 1992).

However, in the present experiments, B cells were cultured in the absence of T cells and macrophages. As such the results do not rule out the possibility that B cells will respond either directly or indirectly when mixed with T cells or macrophages.

4.6.3 Polyclonal Activation of B Cells

The capacity of lectin M to activate B cells for the secretion of Ig was examined by monitoring the amount of IgM and IgG in culture media. There was no significant difference in the IgG and IgM secretion between cultures performed in absence and presence of lectin M. This observation indicates that lectin M was unable to activate murine B-cell for the secretion of immunoglobulin.

4.7 CONCLUSION

Structurally, lectin M is quite similar but not totally identical to artocarpin. For effective comparison of these two closely related lectins, their primary structures are, however, required to be analysed.

The high yield, great stability, inexpensive and easy to obtain properties of lectin M have put it in a better situation than many other lectins as a tool in biomedical and immunological research.

Serum glycoproteins such as α_1-antitrypsin, haptoglobin-β-chain, orosomucoid, transferrin, IgA and IgM were easily detected in ng amount by lectin M as in high-resolution two-dimensional electrophoresis and lectin M-affinoblotting. Thus, lectin M may be used to separate these glycoproteins from other serum proteins, and be a invaluable tool in assessing the modifications of glycan moieties of these glycoproteins. Such modifications are known to have occured in diseases such

as cancer, IgA nephropathy, inflammatory processes and alcoholism. In addition, lectin M-affinity chromatography together with other immobilised-lectins could also be used to generate "fingerprints" of oligosaccharides on the basis of their elution characteristics.

The mitogenic activity shown by lectin M has made it a potential immunological reagent in research on lymphocytes like other lectins. For example: culture media of rat spleen cells stimulated with Con A and PHA have been used as a source of interleukin-2 (Gillis *et al.*, 1980); and jacalin, an excellent choice for obtaining T cell growth activity rich conditioned media from rat spleen cells (Dalmau *et al.*, 1989), has also been used to produce human γ-interferon from T-cell hybridoma (Crane *et al.*, 1984).