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# Effect of Amino Acid Residue and Oligosaccharide Chain Chemical Modifications on Spectral and Hemagglutinating Activity of *Millettia dielsiana* Harms. ex Diels. Lectin

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**Abstract** The effects of modifying the carbohydrate chain and amino acids on the conformation and activity of *Millettia dielsiana* Harms. ex Diels. lectin (MDL) were studied by hemagglutination, fluorescence and circular dichroism analysis. The modification of tryptophan residues led to a compete loss of hemagglutinating activity; however, the addition of mannose was able to prevent this loss of activity. The results indicate that two tryptophan residues are involved in the carbohydrate-binding site. Modifications of the carboxyl group residues produced an 80% loss of activity, but the presence of mannose protected against the modification. The results suggest that the carboxyl groups of aspartic and glutamic acids are involved in the carbohydrate-binding site of the lectin. However, oxidation of the carbohydrate chain and modification of the histidine and arginine residues did not affect the hemagglutinating activity of MDL. Fluorescence studies of MDL indicate that tryptophan residues are present in a relatively hydrophobic region, and the binding of mannose to MDL could quench tryptophan fluorescence without any change in $\lambda_{max}$. The circular dichroism spectrum showed that all of these modifications affected the conformation of the MDL molecule to different extents, except the modification of arginine residues. Fluorescence quenching showed that acrylamide and iodoacetic acids are able to quench 77% and 98% of the fluorescence of tryptophan in MDL, respectively. However, KI produced a barely perceptible effect on the fluorescence of MDL, even when the concentration of $I^-$ was 0.15 M. This demonstrates that most of tryptophan residues are located in relatively hydrophobic or negatively charged areas near the surface of the MDL molecule.

**Key words** *Millettia dielsiana* Harms. ex Diels. lectin (MDL); chemical modification; hemagglutinating activity; circular dichroism; fluorescence quenching

Plant lectins, formerly called phytohemagglutinins, are reversible carbohydrate-binding proteins (or glycoproteins) of non-immuno origin that agglutinate cells and/or precipitate glycoconjugates. All plant proteins that possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide should be included in this group. Plant lectins can be classified into seven lectin families by their evolutionary relationships and sequence similarities, namely legume lectins, chintin-binding lectins, type II ribosome inactive protein and related lectins, monocot mannose-binding lectins, jacalin-related lectins, the amarathin lectin family, and cucurbitaceae phloem lectins [1,2]. Because of their sugar-binding properties, they are useful candidates for the detection of cell-surface carbohydrates [3], biomedical applications [4], host defence [5] and purification of glycoconjugates [6]. It is assumed that lectins play a fundamental biological role in plants since they are found in the organs and tissues of many different species [7].

*Millettia dielsiana* Harms. ex Diels. lectin (MDL), belonging to the legume lectin superfamily, is a dimeric glycoprotein composed of identical subunits with MW of 32 kD. It does not exhibit blood group specificity and oli-

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gosaccharide analysis showed that it contains two oligosaccharide chains [8,9]. Chemical modification of thiol groups, disulfide bonds and tryptophan residues and circular dichroism (CD) spectra of MDL have been studied [10,11].

In this paper, we provide further information on the roles of oligosaccharide chains and tryptophan, histinine, arginine and carboxyl group residues to allow a better understanding of MDL. Fluorescence spectra, circular dichroism spectra and fluorescence quenching were used to study the relationship between conformation and activity of the lectin.

## Materials and Methods

### Materials

*Millettia dielsiana* Harms. ex Diels. lectin was prepared as described previously [8]. Glycinethylester hydrochloride, *N*-bromosuccinimide (NBS) and water-soluble carbodiimide (EDC) were obtained from Acros Organics (Geel., Belgium.) *D*-Mannose, 2,3-butanedione, diethyl pyrocarbonate (DEPC) and $NaIO_4$ were from Serva (Heidelberg, Germany). Acrylamide, KI and iodoacetic acid were from Sigma (St. Louis, USA). All the other reagents were analytical grade.

### Protein determination

The concentration of MDL was determined by using the method of Lowry *et al.* [12], with bovine serum albumin as the standard.

### Hemagglutinating test

The hemagglutinating activity test on MDL was performed by using a two-fold serial dilution in 96-well U-bottomed microtitre plates [13].

### Chemical modification of carbohydrate chain and amino acid residues

Modification of tryptophan residues was carried out according to the method of Spande and Witkop [14]. MDL was dissolved in NaAC (0.1 M, pH 5.0) to 1 mg/ml and divided into five aliquots. Aliquot 1 was taken as the control. Aliquots 3 and 5 contained 0.2 M *D*-mannose (inhibitory sugar). Urea (8 M) was added into aliquots 4 and 5. The modification was carried out at 20 °C. NBS (10 μl 10 mM) was added into aliquots 2–5 every 5 min. The number of tryptophan residues was calculated as described by Spande and Witkop [14]. The samples were then dialysed against deionized water to remove the excess reagent, and then the hemagglutinating activity test was carried out.

Oxidation of the carbohydrate chains of MDL with $NaIO_4$ was carried out according to the method of Lotan and Debray [15]. MDL was dissolved in NaAC (0.02 M, pH 5.0) to 4 mg/ml, 1.5 ml 10 mM $NaIO_4$ was added at 0 °C and the absorbance was recorded at 222 nm. The excess reagent was removed by dialysis as mentioned above.

Histidine residues were modified according to the procedure of Miles [16]. The samples were dialyzed to remove the excess reagent and tested for their hemagglutinating activity.

Modifications of aspartic acid and glutamic acid residues were carried out according to the method of Goldstein [17]. In addition, the MDL was treated with EDC in the presence of *D*-mannose (0.2 M). The excess reagent was removed by dialysis and the hemagglutinating activity was assayed.

Arginine residues were modified according to the method of Lu *et al.* [18]. MDL (1 mg/L) reacted with 50 mM 2,3-butanedione in sodium borate buffer (25 mM, pH 8.5) for 2 h at 25 °C. The hemagglutinating activity of modified MDL was assayed after the removal of the excess reagent by dialysis.

### Spectroscopic measurements

Fluorescence spectroscopy was carried out using a spectrophotofluorometer (Model 4500, Hitachi Ltd., Tokyo, Japan). The samples were excited at 280 nm and 295 nm, and then the emission spectra were recorded.

CD spectra were obtained using a Jasco-500C spectropolarimeter (Jasco, Tokyo, Japan) at the wavelength ranges of 320–250 nm and 250–190 nm.

### Fluorescence quenching

Tryptophan fluorescence quenching of MDL was studied according to the method described by Lehrer *et al.* [19] and Eftink *et al.* [20].

## Results and Discussion

Modifications of crucial amino acid residues in the active sites of lectins with group-specific reagents may bring about changes in the biological character of lectins. This change can be monitored by analysis of the hemagglutinating activity and conformation of the modified lectin. Thus, chemical modification provides a useful approach for the identification of amino acid residues present in the

**Table 1 Effect of chemical modification on the hemagglutination activity of MDL**

| Aliquot No. | Chemicals | Modified part | Hemagglutinating activity (%) |
|---|---|---|---|
| 1 | Native MDL | – | 100 |
| 2 | $NaIO_4$ | Carbohydrate-chain | 100 |
| 3 | DEPC | Histidine | 100 |
| 4 | EDC | Carboxyl | 20 (without *D*-mannose) |
| | | | 100 (with *D*-mannose) |
| 5 | 2,3-butanedione | Arginine | 100 |
| 6 | NBS | Tryptophan | 0 (without *D*-mannose) |
| | | | 100 (with *D*-mannose) |

functional or active site of lectins [21]. The results of chemical modification of MDL are listed in **Table 1**. Modification of tryptophan residues resulted in a complete loss of activity; 3.6 and 3.8 tryptophan residues were modified in the absence or presence of urea. A plot of the percentage residual activity versus the number of tryptophan residues modified is shown in **Fig. 1**. These data suggest that about two tryptophan residues are essential for hemagglutinating activity. In contrast, when mannose was added before the modification, only 2.1 and 2.3 tryptophan residues were modified. A hemagglutinating activity assay carried out after this modification showed that the activity of MDL was intact. These results suggest that about two tryptophan residues are located in the carbohydrate-binding site and are involved directly in mannose binding. Modification of the carboxyl groups caused an 80% reduction in hemagglutinating activity, but the modification was inhibited by 0.2 M *D*-mannose. These results suggest that carboxyl groups are involved in the carbohydrate-binding site of MDL. The modification of carbohydrate chains, and histidine and arginine residues did not affect the hemagglutinating activity of the lectin. The results of this study rule out the possibility that carbohydrate chains and these residues are involved in the carbohydrate-binding site.

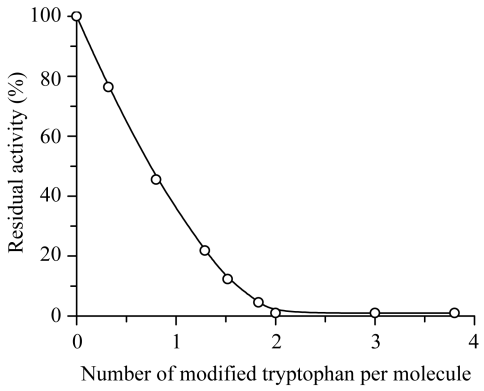


**Fig. 1 Effect of the modification of tryptophan residues on the hemagglutinating activity of MDL**

Native MDL exhibited a fluorescence emission maximum ($\lambda_{max}$) at 332 nm upon excitation at both 280 nm and 295 nm, indicating the presence of tryptophan in a relatively hydrophobic region [22,23]. MDL with modified tryptophan and carboxyl groups showed a 20% decrease in the relative fluorescence intensity on the addition of the mannose, which indicates that the binding of mannose to MDL causes a change in the tryptophan environment; however, there was no significant change in the $\lambda_{max}$ of native MDL and the mannose-MDL complex, which may indicate that there is no change in the hydrophobic environment of tryptophan upon binding of mannose. Decreases in the relative fluorescence patterns of tryptophan emission with modification of the tryptophan and carboxyl groups residues are shown in **Figs 2**, **3** and **4**.

Changes in the fluorescence spectra when MDL was modified by various reagents are shown in **Fig. 5**. Modification of the carbohydrate chain led to a decrease in the intrinsic fluorescence upon excitation at 280 nm and 295 nm (**Fig. 5**). Decreases in the fluorescence intensity, as well as the maximum blue-shift at 280 nm, may mainly result from the carbohydrate chain oxidation. The results suggest that the carbohydrate chains might be required for maintaining the conformation of MDL. In some cases, thiol groups and disulfide bonds in MDL may be affected by $NaIO_4$ [16]. Decreases in the fluorescence intensity might also partly result from the oxidation of thiol groups and disulfide bonds. Modification of histidine residues led to a decrease in relative intensity at 280 nm and 295 nm.

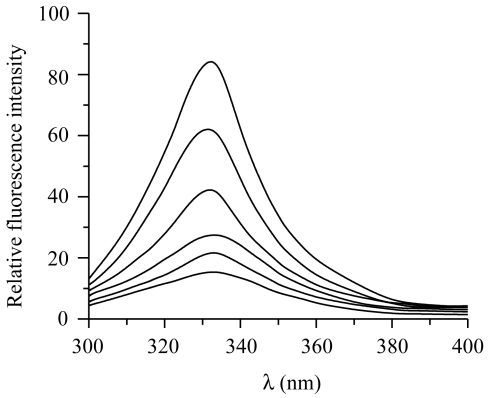


**Fig. 2 Fluorescence spectrum of MDL with different concentrations of NBS (without *D*-mannose)**

Curves from top to bottom represent the fluorescence spectra after the addition of 0, 10, 20, 30, 40 and 50 μl of 10 mM NBS, respectively. Excitation was performed at 280 nm.

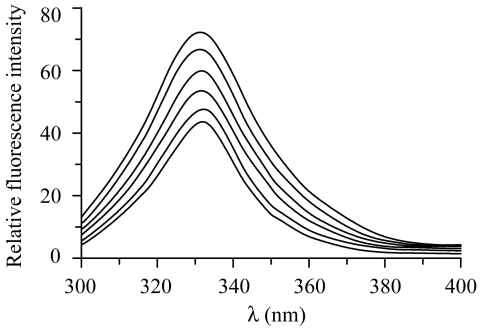


**Fig. 3 Fluorescence spectrum of MDL with different concentration of NBS (with 0.2 M *D*-mannose)**

Curves from top to bottom represent the fluorescence spectra after the addition of 0, 10, 20, 30, 40 and 50 μl of 10 mM NBS, respectively. Excitation was performed at 280 nm.

This decrease is mostly due to the modification of histidine residues. Although DEPC is specific for histidine at and around neutral pH, it also reacts to a lesser extent with thiol groups, and lysine and arginine residues [16], and the decrease in fluorescence intensity might also partly result from the modification of these residues. Modification of carboxyl and arginine residues did not cause any significant change in the spectra upon excitation at either 280 nm or 295 nm, so the microenvironment around tryptophan was not affected by the modification.

The CD spectra of MDL modified by various reagents in the far- and near-UV regions are shown in **Fig. 6**. A

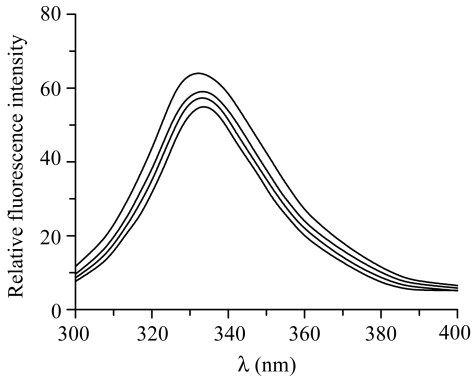


**Fig. 4 Fluorescence spectrum of EDC modified MDL in the absence or presence of 0.2 M *D*-mannose**

Curves from top to bottom represent the fluorescence spectra for native MDL, native MDL+*D*-mannose, EDC-modified MDL+*D*-mannose, and EDC-modified MDL, respectively. Excitation was performed at 280 nm.

negative peak with a minimum at 216 nm at the far-UV CD spectrum of native MDL indicates a characteristic β-sheet conformation [24]. The spectrum in the near-UV region exhibited a negative band at 282 nm and two negative shoulders centered at 260–275 nm and 295 nm [10, 11]. Modification of arginine residues did not lead to significant changes in the far- and near-UV CD spectra of MDL (**Fig. 6**). It showed that the modification of arginine residues did not affect the conformation of the MDL molecule. The double negative peaks resulted from the modification of the carbohydrate chain and histidine residues, which are representative features of the α-helix in the far-UV region [24]. However, there were no significant changes in the near UV region. Carbohydrate chains play important roles in controlling conformation maturation, and maintaining the stability and activity of proteins [25, 26]. In some enzymes, sugar chains have no involvement in the activity of enzymes [27,28]. Studies suggest that all sugar chains of cationic peanut peroxidase (cPrx) are required for the stable conformation of cPrx, but one of the three sugar chains is not essential for the catalytic activity of the enzyme [29]. As for MDL, sugar chains may be essential for stable conformation rather than activity, since modification of the carbohydrate chain did not affect the essential conformation of the active center. We found that the modification of histidine residues resulted in a marked change of CD spectra, so histidine residues must play an important role in maintaining the conformation of MDL. However, modification of histidine residues did not affect the hemagglutinating activity (**Table 1**), which suggests that the conformational change in histidine- modified MDL

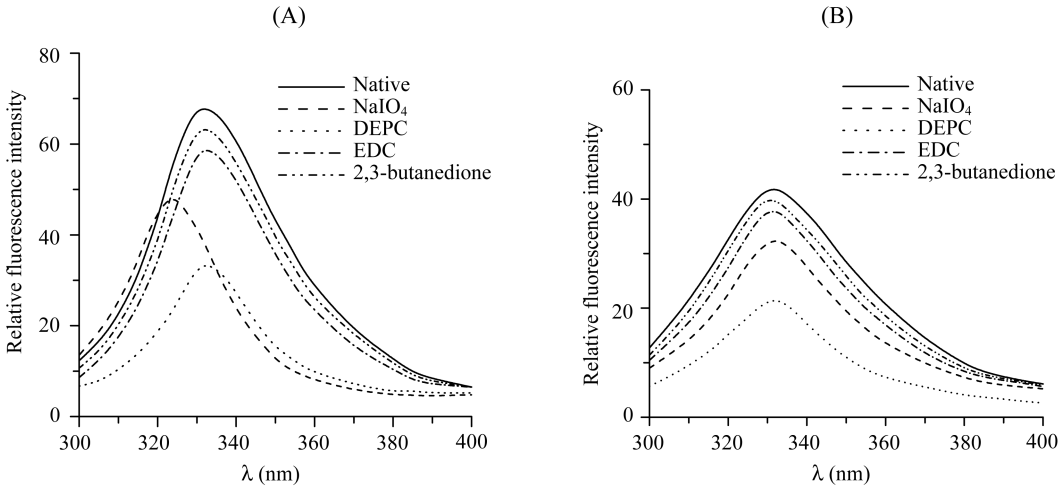


**Fig. 5 Fluorescence spectra of MDL with different modifications**

(A) Excitation was performed at 280 nm. (B) Excitation was performed at 295 nm.

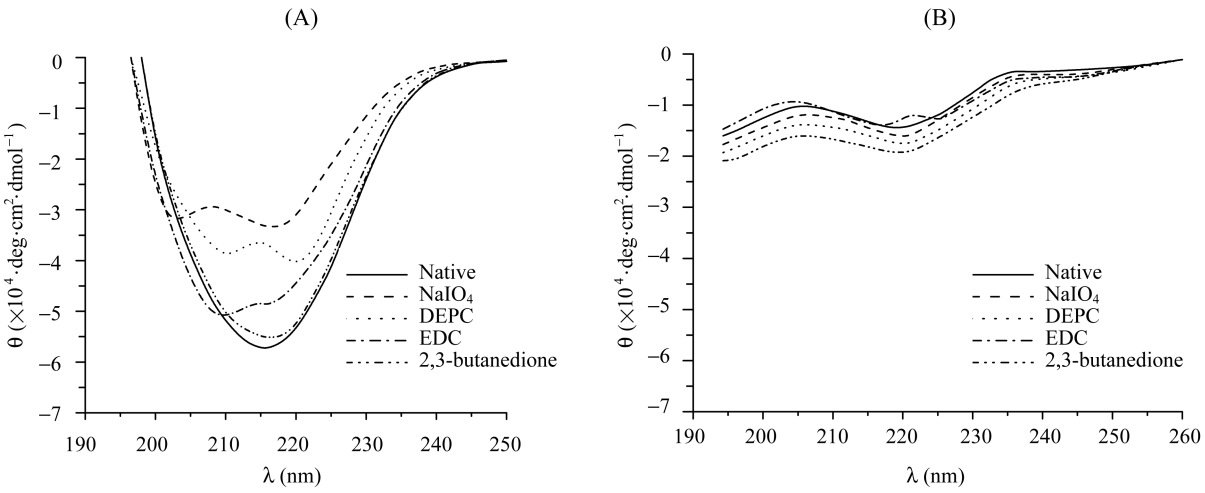


**Fig. 6 CD spectra of MDL modified by various reagents**

(A) Far-UV CD spectra. (B) Near-UV CD spectra.

did not destroy the essential conformation of the activity center, so the hemagglutinating activity remained intact. Modification of carboxyl residues led to significant changes in the CD spectra of MDL, both in the far- and near-UV regions (**Fig. 6**). The negative peak blue-shifted to 210 nm and the absolute value of the maximum declined. In the near-UV CD spectrum, the negative peak also blue-shifted. These changes reflect the conformational changes of MDL. The loss of hemagglutinating activity of the carboxyl-modified MDL might be due to the modification of carboxyl groups rather than a structural change, since the activity of the control remained intact and the change in its CD spectrum was almost the same as the carboxyl-modified MDL (data not shown). The change in the CD spectrum and the secondary structure of the carboxyl-modified MDL are probably due to the extreme modification buffer.

In the quenching experiment, data were analyzed by the Stern-Volmer equation [**Equation (1)**] and the modified Stern-Volmer equation [**Equation (2)**] [30]:

$$F_0/F=1+K_Q[Q] \quad (1)$$

$$F_0/(F_0-F)=f_m^{-1}+(K_a f_m)^{-1}[Q]^{-1} \quad (2)$$

where $F_0$ and $F$ are the respective fluorescence intensities,

corrected for dilution, in the absence and in the presence of a quencher, [*Q*] is the resultant quencher concentration. $K_Q$ is the Stern-Volmer quenching constant of MDL for a given quencher, $f_m$ refers to the fraction of the corresponding Stern-Volmer quenching constant. Quenched by acrylamide at five different concentrations, the emission spectra of MDL excited at 295 nm are shown in **Fig. 7**. As **Fig. 8** shows, a linear plot of $F_0/F$ vs. [*Q*] was obtained and $K_Q$=2.0. According to Equation (2), $f_m$ was determined to be 0.77, which meant that 77% of the fluorescence of tryptophan residues in MDL could be quenched. Acrylamide quenches the fluorescence of indole derivatives predominately by a collisional process, and is insensitive to the change surrounding tryptophan fluorophores. Thus, it can be used to assess the degree of the exposure of tryptophan residues in protein. The close approach of fluorophore and quencher can be prevented by steric factors as well as by charged factors. Since a charged and hydrated quencher such as iodide is sensitive to local charge effects, quenching by this molecule can reveal the charged state of the local environment around tryptophan fluorophores [31]. As for MDL, the ionic quencher, iodide, almost didn't completely quench the total fluorescence of MDL when $I^-$ was 0.15 M. This suggests that the tryptophan residues are located in a relatively negatively charged environment, which may be due to the tryptophan residues being found in the close vicinity of a carboxylic acid residue [23]. Our hemagglutinating activity assays indicated the presence of carboxylic groups at the active site of MDL. Similarly, fluorescence quenching by iodoacetic acid is shown in **Fig. 9** and **Fig. 10** ($K_Q$=0.83 and $f_m$=0.98). From the results above, we conclude that nearly all tryptophan residues in MDL are located in the shallow channels of the molecule surface, and most of them are located in the relatively hydrophobic or negatively charged areas, where $I^-$ could not easily approach.

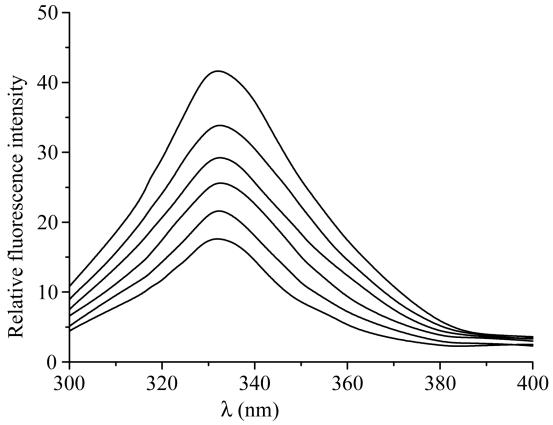


**Fig. 7 Quenching of MDL intrinsic fluorescence spectra by acrylamide**

Excitation wavelength was 295 nm. Concentrations of acrylamide from top to bottom were 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 M, respectively.

## Conclusions

The carbohydrate-binding site in legume lectin consists

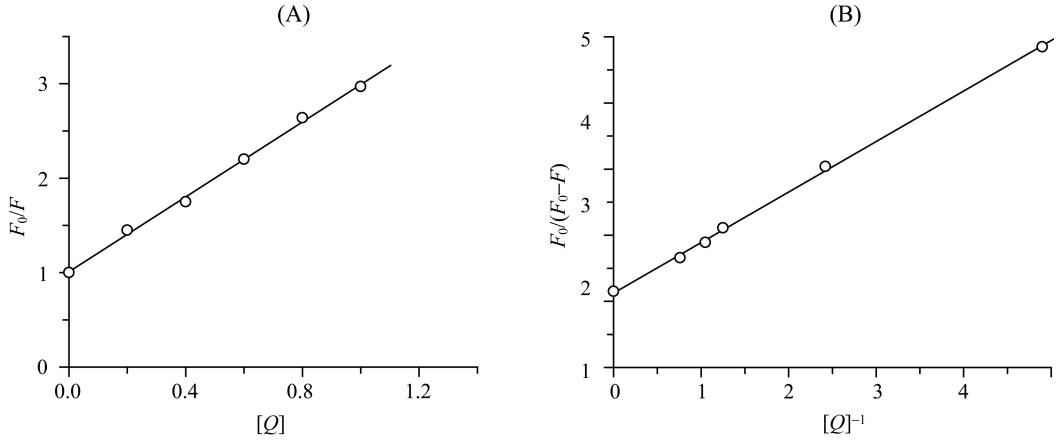


**Fig. 8 Quenching of MDL intrinsic fluorescence by acrylamide**

(A) The plot of $F_0/F$ versus [*Q*]. (B) The plot of $F_0/(F_0-F)$ versus 1/[*Q*].

of a core of a small number of well-conserved residues surrounded by a variable perimeter. These conserved residues include the triad Asp/Gly/Asn that makes key hydrogen bonds to the sugar and an aromatic residue that stacks onto the sugar ring [32]. In some legume lectins, such as soybean agglutinin, *Dolichos biflorus* seed lectin, and *Griffonia simplicifolia* lectin IV, tryptophan residues are essential for carbohydrate recognition and are involved in the carbohydrate-binding site directly [33]. Tryptophan residues are also crucial for the hemagglutinating activity of MDL [10]. In the present study, all of the tryptophan residues could be modified in the absence or presence of denaturant, suggesting that all the tryptophans are located on the near surface or in the shallow channels of the MDL surface [11], since well-refined X-ray structures of protein-carbohydrate complexes have elucidated that the carbohydrate-binding sites of plant lectins are mainly located in shallow grooves close to the protein surfaces [34]. The hemagglutinating activity of the tryptophan-modified lectin remained unchanged and two tryptophan residues were protected in the presence of *D*-mannose, thus two tryptophan residues are located in the carbohydrate-binding site of the shallow channel near the surface of MDL. The modification of carboxyl groups led to an 80% reduction in the hemagglutinating activity of MDL, but the activity was also intact when modification was performed in 0.2 M *D*-mannose. Therefore, carboxyl groups were important for the carbohydrate binding activity, and some of the glutamic and aspartic acids located in the carbohydrate-binding site were directly involved in mannose binding. The evidence for the presence of glutamic acid and aspartic acid residues in the carbohydrate binding site of legume lectins, such as *Concanavalin* A, *Galanthus nivalis* agglutinin, *Helianthus tuberosus* agglutinin, *Ulex europaeus* lectin II and *Erythrina corallodendron* lectin, has been reported [35–37]. The results of the present study suggest that tryptophan and carboxyl groups are essential for carbohydrate binding activity and are involved in carbohydrate binding sites. In addition, the carbohydrate chain and histidine residues are important for maintaining the conformation of MDL, but not for the hemagglutinating activity of the lectin. Fluorescence and quenching studies show that most of the tryptophan residues are located in relatively hydrophobic or negatively charged areas near to the MDL surface. Future studies would focus on the amino acid composition in the carbohydrate-binding site of MDL. Techniques that could be used are molecular cloning, site-directed mutagenesis and even X-ray crystallography. These studies would contribute immensely to our understanding of the features of the atomic interactions between lectins and carbohydrates.

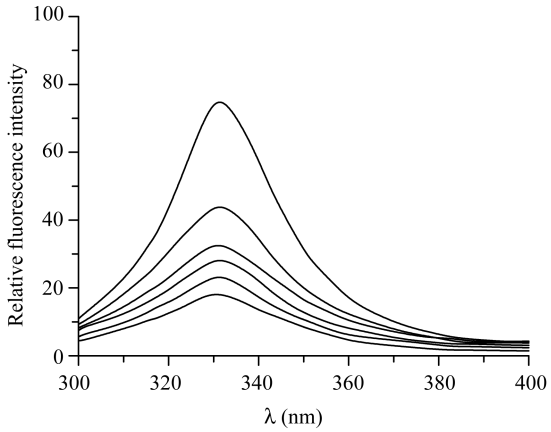


**Fig. 9 Quenching of MDL intrinsic fluorescence spectra by iodoacetic acid**

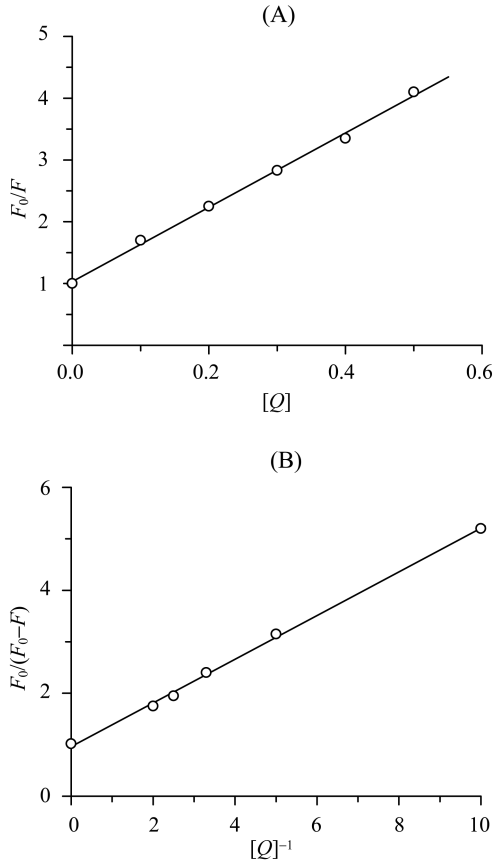


**Fig. 10 Quenching of MDL intrinsic fluorescence by iodoacetic acid**

(A) The plot of $F_0/F$ versus [$Q$]. (B) The plot of $F_0/(F_0-F)$ versus 1/[$Q$].

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