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Involvement of Tyrosine, Histidine and Cystein Residues in the Saccharide Binding Site of Mulberry Seed Lectins

[1]Tanzima Yeasmin, [2]Abul Kashem Tang, [1]Anowar Hossain and [1]Nurul Absar
[1]Department of Biochemistry and Molecular Biology, Rajshahi University, Rajshahi-6205, Bangladesh
[2]Department of Applied Nutrition and Food Science, Islamic University, Kustia, Bangladesh

Abstract: This study deals with the effect of different chemical modifying agents on the mulberry seed protein, namely MSL-1 and MSL-2, to obtain information on the structure-function relationship of these lectins. Mulberry seed lectins, MSL-1 and MSL-2, having specificity for D-galactose were subjected to various chemical modification in order to obtain information about the amino acid residues responsible for their carbohydrate binding property. The modifying reagents used are mild and very much selective for modification of specific groups. Modification of tyrosine, histidine and thiol, groups led to a complete or significant loss of their activities, indicating the involvement of these amino acid residues in the saccharide binding property of the lectins. However, modification of lysine, arginine and trypotophan residues had no such noticeable effect on the biological activities of these two lectins. These findings are very similar to those reported for other lectins that bind D-galactose and also fit the general trend in other lectins.

Key words: Mulberry seed lectins, saccharide binding site(s), chemical modification

INTRODUCTION

Lectins are divalent or multivalent carbohydrate binding proteins that bind to the carbohydrate moiety of the cell membrane and mediated a variety of biological effects (Goldstein and Hayes, 1978; Lis and Sharon, 1973). The carbohydrate binding site(s) of a lectin contain amino acids with side chains that contribute to the union of specific carbohydrate as ligands with the lectin, analogous to enzyme-substrate association. The hemagglutinating property of a lectin is attributed to their interaction with specific sugar. Chemical modification with amino acid modifying reagents provides a general approach for identification of the amino acid residues in the active site of protein or lectins (Ray and Chatterjee, 1989; Sultan *et al*., 2004; Nadimpalli, 1999). Mulberry seeds crude extract contains three lectins of which showed specific binding with the galactose and glactose containing saccharides (Yeasmin *et al*., 2001).

This study deals with the effect of different chemical modifying agents on the mulberry seed proteins, namely MSL-1 and MSL-2, with a view to obtain information on the structure-function relationship of these lectins.

MATERIALS AND METHODS

Mulberry seeds were collected from the experimental plot of Bangladesh Sericulture Research and Training Institute, Rajshahi in the month of September-December and brought to the Protein Research Laboratory, University of Rajshahi and Bangladesh for experimental purpose.

Corresponding Author: Tanzima Yeasmin, Department of Biochemistry and Molecular Biology, Rajshahi University, Rajshahi-6205, Bangladesh Tel: 0721-750041-4109, 0721-750041-3758 Fax: 0721-750064

Mulberry seed lectins were purified from seed as described previously (Yeasmin *et al*., 2001). Sephadex 4B and citraconic anhydride were product of Sigma (St. Louis, USA). N-acetlyimidazole, N-bromo-succinimide, Diethyl pyrocarbonate, 5,5′-Dithiobis and p-nitrophenyl glyoxal were perched from Fluka Biochemica, Switzerland while acetic anhydride and glyoxalamine were perched from British Drug House.

Protein Measurement

Protein concentration of MSL-1 and MSL-2 were estimated by monitoring absorbance at 280 nm as reported earlier (Yeasmin *et al*., 2001).

Assay Procedure

Hemagglutintion assays were performed using 4% albino rat red blood cells in phosphate buffer saline, pH-7.2 following the method as described by Lin *et al*. (1981).

Chemical Modification of MSL-1 and MSL-2

Acetylation

Acetylation of MSL-1 and MSL-2 was performed according to the methods of Rice and Etzlar (1974). The modified protein was dialyzed exhaustively against PBS at 4°C. De-O-acetylation of modified lectin was made with respect to hydroxyl amine (upto 1 M) by addition of an equal volume of 2 M hydroxyl amine-HCl at pH 7.5 and the pH was adjusted with 0.1 M NaOH. The solution was mixed uniformly and left at 20°C for 15 min with occasional string and then dialyzed against distilled water at 0-4°C (Ashfold *et al*., 1981).

Citraconylation

The method of Habeeb *et al*. (1958) was used lectin solution (0. 6-1.0 mg mL^{-1}) in Tris-HCl buffer, pH-8.4 was mixed with slow constant stirring, approximately 400 fold molar excess of the reagent at 4°Cfor 1 h. The solution then dialyzed extensively against PBS, pH 7.2 at 0-4°C.

Determination of the Extent of Modification

The extant of acetylation and citraconylation was determined by measuring the remaining amino and phenolic hydroxyl groups by reaction with 2,4,6-trinitiomethane respectively and comparing the values obtained with those of native lectin (Habeeb *et al*., 1967).

Treatment with P-nitrophenyl Glyoxal

Arginine residues of the lectins were modified with p-nitrophenyl glyoxal following the method as described by Yamasaki *et al*. (1981).

Treatment with Diethyl Pyrocarbonate

MSL-1 and MSL-2 were treated with diethyl pyrocarbonate as reported by Muhlard *et al*. (1967). The number of histidine residues modified was calculated from the difference molar extinction at 240 nm ($\Delta\sum = 3{,}200\ cm^{-1}\ M^{-1}$)for carbethoxy histidine (Miles, 1977).

Acetylation with N-Acetylimidazole

The acetylation was carried out as described by Riordan *et al*. (1965). The number of O-acyl groups introduced was determined from the change in absorbance at 278 nm. De-O-acetlyation of the acetylated lectin with 1 M hydroxylamine-HCl was performed following the method as described (Ashfold *et al*., 1981).

Treatment with N-Bromosuccinamide

MSL-1 and MSL-2 was modified with NBS as described by Spande *et al*. (1966). The number of tryptophan residues oxidized was calculated according Spande and Witkop (1967).

Treatment with 5,5′-Dithiobis-2-nitrobenzene (DTNB)

The method of Ellman (1959) was used for modification of -SH groups with DTNB. An extinction co-efficient of 13,600 M^{-1} cm^{-1} is used to calculate the number of -SH groups modified (Means and Feeney, 1971).

RESULTS

Acetylation of MSL-1 and MSL-2 with Acetic Anhydride

Lysine recidues including the terminal α-amino group and the phenolic hydroxyl group such as tyrosine residues were modified by acetic anhydride (Dixon and Perham, 1968). It is evident from the results that the hemaggluting activities as well as binding abilities to Sepharose 4B were lost completely after acetylation of the lectin with acetic anhydride (Table 1). The results also indicated that, nineteen amino groups and five tyrosine residues in MSL-1 while sixteen amino groups and four tyrosine residues in MSL-2 are associated with the activities. Further de-o-acetylation of the modified lectins on treatment with 1 M-hydroxylamine at pH 7.5, regenerated about four and three tyrosine residues of MSL-1 and MSL-2 respectively, which at the same time the biological activities of the modified lectins were also recovered to about 75-80%. It may also be mentioned that a significant number of tyrosine residues of both the lectins as well as some amino groups of MSL-1 were protected from acetylation when the modification reaction was done in the presence of specific saccharide, 0.2 M galactose and the modified lectins retained about 70-80% of their activities.

Citraconylation of MSL-1 and MSL-2

Reaction of protein with citraconic anhydrate results specifically in the incorporation of citraconyl groups to free amino groups (Habeeb *et al*., 1958). Table 2 shows the relationship between biological activities and the average number of citraconyl groups introduced in modified MSL-1 and MSL-2. From these results it is clear that the hemagglutinating activity as well as binding ability to

Table 1: Effects of modification with acetic anhydride on hemagglutinating activity and carbohydrate binding properties of MSL-1 and MSL-2

Protein	Conditions	Types of group modified	No. of residues modified per mole of lectin	Hemagglutinating activity (%)	Binding ability to sepharose 4B (%)
MSL-1	None	-	-	100	100
MSL-2	None	-	-	100	100
MSL-1	Acetic anhydride	Tyr*	5.05±0.2	0	0
		NH_2†	19.10±0.2		
MSL-2		Tyr*	4.02±0.3	0	0
		NH_2†	16.00±0.3		
MSL-1	Acetic anhydride in presence of 0.2M Galactose	Tyr*	1.40±0.2	80	80
		NH_2†	15.30±0.2		
MSL-2		Tyr*	0.75±0.2	75	80
		NH_2	15.85±0.2		
MSL-1	Acetic anhydride followed by 1 M hydroxyl amine, pH 7.5	Tyr*	0.80±0.2	80	80
		NH_2†	18.70±0.2		
MSL-2		Tyr*	1.65±0.2	70	80
		NH_2	4.00±0.2		

*No. of residues modified as determined from the change in absorbance at 276 nm according to Riordan *et al*. (1965).
†No. of residues modified as calculated by the method of Habeeb (1967)

Table 2: Effects of citraconylation on hemagglutinating activity and carbohydrate binding properties of MSL-1 and MSL-2

Protein	Conditions	Types of group modified	No. of residues modified per mole of lectin	Hemagglutinating activity (%)	Binding ability to sepharose 4B (%)
MSL-1	None	-	-	100	100
MSL-2		-	-	100	100
MSL-1	Citraconic anhydride	NH_2^+	19.60±0.30	70	70
MSL-2		NH_2^+	16.20±0.30	65	75
MSL-1	Citraconic anhydride	NH_2^+	18.03±0.15	75	70
MSL-2	(In presence of 0.2 M galactose)	NH_2^+	15.30±0.15	70	75

+No. of residues modified as calculated by the method of Habeeb (1967)

Table 3: Effects of acetylation with N-acetylimidazole on hemagglutinating activity and carbohydrate binding properties of MSL-1 and MSL-2

Proteins	Treatments	*N-acetylation (mole/mole protein)	†O-Tyrosyl acetylation (mole/mole protein)	Hemagglutinating activity (%)	Binding ability to sepharose 4B (%)
MSL-1	-	0	0	100	100
MSL-2		0	0	100	100
MSL-1	Molar ratio:	15.25±0.15	5.87±0.2	0	0
MSL-2	N- acetylimidazole protein 30:1	15.20±0.15	2.92±0.2	0	0
MSL-1	Acetylation	12.50±0.20	2.09±0.2	80	90
MSL-2	(In presence of 0.2 M galactose)	12.05±0.2	1.05±0.2	75	85
MSL-1	Deacetylation by 1 M	14.80±0.2	1.50±0.2	75	85
MSL-2	hydroxyl-amine, pH 7.5	15.08±0.2	0.50±0.2	80	85

*No. of residues modified as determined from the change in absorbance at 276 nm according to Riordan *et al.* (1965), †No. of residues modified as calculated by the method of Habeeb (1967)

Sepharose 4B of the lectins were reduced moderately after citraconylation. Further, in the presence of specific saccharide, galactose about one amino group in both the lectins are protected from citraconylation but the biological activities of the lectins were also found to be almost same as that observed if citraconylation was carried out in the absence of galactose.

Effect of Arginine Residue Modification

Arginine residues of protein in aqueous solution react with glyoxal between pH 8.0-9.0 but the nature of the derivative formed is unknown (Nakaya *et al.*, 1967). The present findings clearly indicated that treatment of MSL-1 and MSL-2 with 10% p-nitrophenyl glyoxal did not produced any noticeable effects on the biological activities of the lectins (Data, not given).

Effect of Acetylation with N-Acetylimidazole

Acetylation of proteins with N-acetylimidazole results in a preferential modification of tyrosyl residues (Riordan *et al.*, 1965). As shown in Fig. 1A and B during acetylation the UV-absorption spectra of MSL-1 and MSL-2 were decreased around 278 nm. Further, the data in Table 3 show that such treatment of MSL-1 and MSL-2 resulted in modification of about six and three tyrosine residues, respectively as calculated from the changes in absorbance at 278 nm accompanying the reaction (Riordan *et al.*, 1965). In both the cases this modification reduced strongly the cytoagglutinating activity as well as binding ability to Sepharose 4B. These effects were almost completely reversed by subsequent treatment with hydroxylamine which is know to regeneration free tyrosyl residues (Simpson *et al.*, 1963). It may be mentioned that in this treatment, amino groups of both the lectins are also modified. Significant protection effect of tyrosine residues (about four in MSL-1 and about two in MSL-2) as well as amino groups (About three in both MSL-1 and MSL-2) were found when the modification was carried out in the presence of specific saccharide, 0.2 M galactose and the modified lectins also retained its activities almost completely.

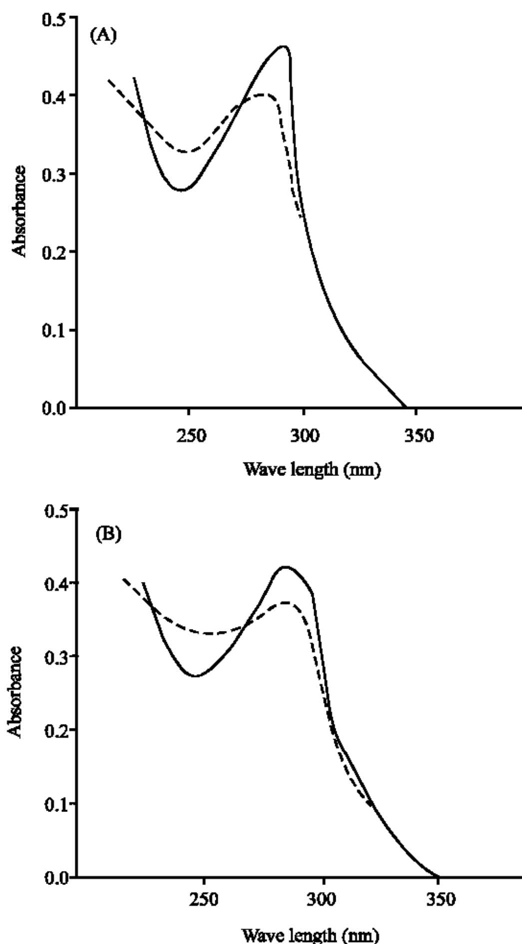

Fig. 1: Acetylation of tyrosyl residues in MSL-1 (A) and MSL-2 (B) with N-acetylimidazole. Acetylation were carried out with 30 fold molar excess of the reagent. One microliter acetic anhydride were added to 0.8-1.0 mg of lectins in 2.5 mL of 50% saturated sodium acetate buffer, pH 8.3 at 0°C with continuos stirring. After 1 h, the treated sample was then dialyzed against distilled water following by dialysis against 5 mM phosphate buffer, pH 7.6 at 4°C (_______) spectra of the native proteins and (--------) spectra of acetylated protein after removal of excess N-acetylimidazole by gel filtration

Oxidation of Tryptophan with NBS

As demonstrate in Fig. 2A and B NBS-oxidation of MSL-1 and MSL-2 were greatly influenced by pH. It is evident that extent of tryptophan oxidation increased with lowing pH and about three tryptophan residues per molecule were oxidized. To oxindolealanine at pH 6.0. Whereas five tryptophan residues per molecule were modified at pH 3.5 in both the lectins (Fig. 2 and Table 4). Significantly, both the lactins retained about 70-75%, biological activities even after oxidation of five tryptophan residues per molecule at pH 4.5. Further, very slight protective was observed when the lectins were modified in the presence of specific saccharide, 0.2 M galactose. On the other hand, eleven tryptophan residues per molecule of MSL-1 and ten tryptophan residues

Table 4: Biological activities of NBS-oxidized MSL-1 and MSL-2

Proteins	Treatments	pH	No. of residues modified per mole of lectins	Hemagglutinating activity (%)	Binding ability to Sepharose 4B (%)
MSL-1	-	-	0.0	100	100
MSL-2	-	-	0.0	100	100
MSL-1	NBS	6.0	3.4±0.3	80	80
MSL-2			3.2±0.3	85	85
MSL-1	NBS	5.5	4.1±0.3	75	75
MSL-2			4.2±0.3	75	75
MSL-1	NBS	4.5	4.9±0.3	70	70
MSL-2			4.8±0.3	70	75
MSL-1	NBS	3.5	5.0±0.2	65	70
MSL-2			5.2±0.2	65	75
MSL-1	NBS+0.2 M galactose	4.5	4.7±0.2	70	85
MSL-2			4.7±0.2	80	75
MSL-1	NBS+8 M urea	4.5	11.1±0.15	0	0
MSL-2			10.3±0.15	0	0

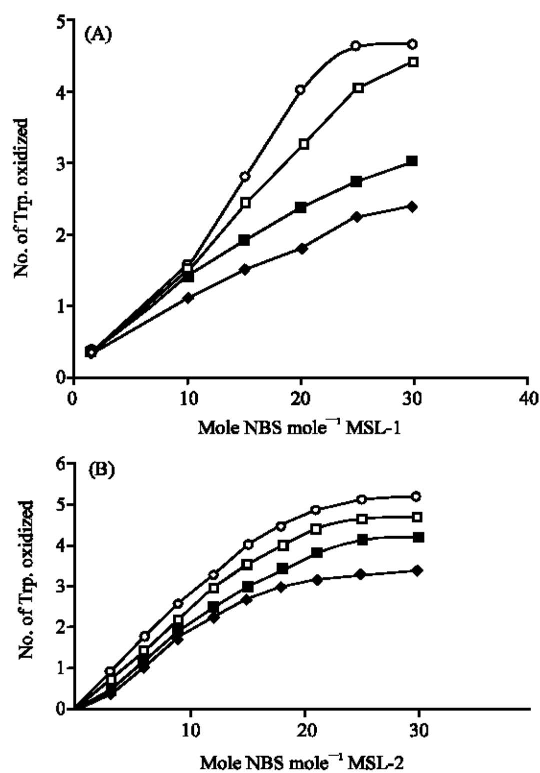

Fig. 2: NBS oxidation of tryptophan residues in MSL-1 (A) and MSL-2 (B) at various pH's. The concentration of MSL-1 and MSL-2 were 4.6×10^{-6} and 4.7×10^{-6} in 0.1 M buffer of respective pH. The reaction was followed at 20°C and the following buffer were used throughout the experiment. Sodium acetate: pH 4.0-0.5 and NaH_2PO_4-Na_2HPO_4: pH 6.0. (○) pH 3.5, (□) pH 4.5, (■) pH 5.5 and (♦) pH 6.0

per molecule of MSL-2 were modified when the lectins were modified with NBS at pH 4.5 in the presence of 8 M Urea and the resulting modified lectins at the same time lost their biological activities completely.

Modification of Histidine Residues with DEPC

The preferential modification of histidine residues with DEPC has been well established (Miles, 1977) Fig. 3A and B shows the ultraviolet difference spectra of DEPC treated MSL-1 and MSL-2, respectively at different time intervals. The spectrum has a maximum at 240 nm, owing to the formation of N′-ethoxyl formyl histidine (Miles, 1977), while no noticeable absorption change is observed around 280-300 nm. As shown in the Fig. 3, nearly 75% modification reaction of the lectins was completed within 17-18 min. Thereafter, the reaction was reached near a plateau. From, the variation of the intensively at 240 nm, the number of histidine residues modified with DEPC was calculated at pH 7.2, eight and six histidine residues in MSL-1 and MSL-2 were modified with DEPC but in the presence of specific saccharide, 0.02 M galactose under identical condition, four and two

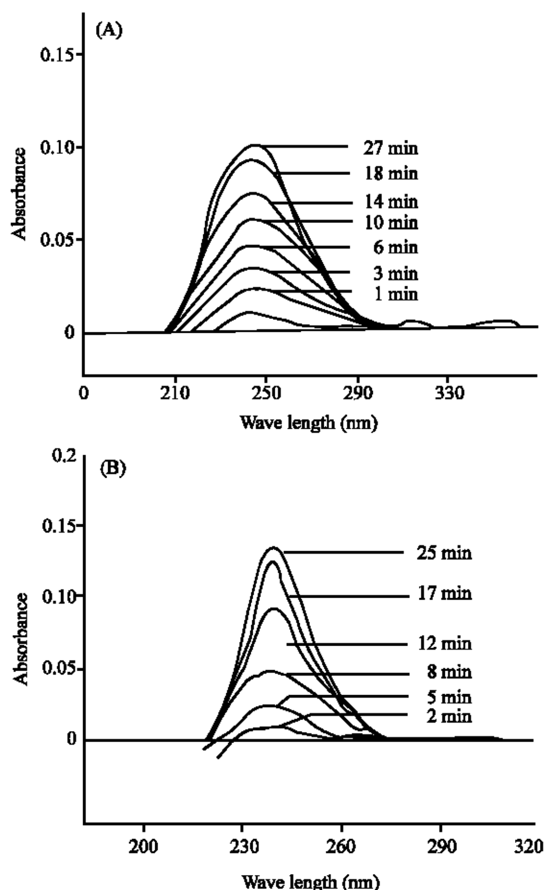

Fig. 3: Ultraviolet difference absorption spectra of DEPC modified mulberry seed lectins A: MSL-1 and B: MSL-2. 4.4×10^{-6} M and $4.0\times10\times^{6}$ M lectins, were placed in the reference and sample cuvette in 20 mM sodium acetate phosphate buffer pH 7.2, at 20°C. The spectra were run at different time intervals after addition of 5 μL DEPC solution to the sample cuvette. The reference cell contained all ingredients except DEPC but receives equal amount of buffer

Table 5: Biological activities of DEPC treated MSL-1 and MSL-2

Proteins	Chemical treatment	No. of His residues modified (moles/mole protein)	Hemagglutinating activity (%)	Binding to Sepharose 4B (%)
MSL-1	-	0	100	100
MSL-2				
MSL-1	DEPC	8.25±0.30	0	0
MSL-2		5.89±0.30	5	
MSL-1	DEPC	4.20±0.25	90	90
MSL-2	(in presence of 0.2 M galactose)	3.60±0.25	80	85
MSL-1	DEPC followed by	0.84±0.25	95	95
MSL-2	hydroxyl amine, pH -7.5	0.30±0.25	95	95

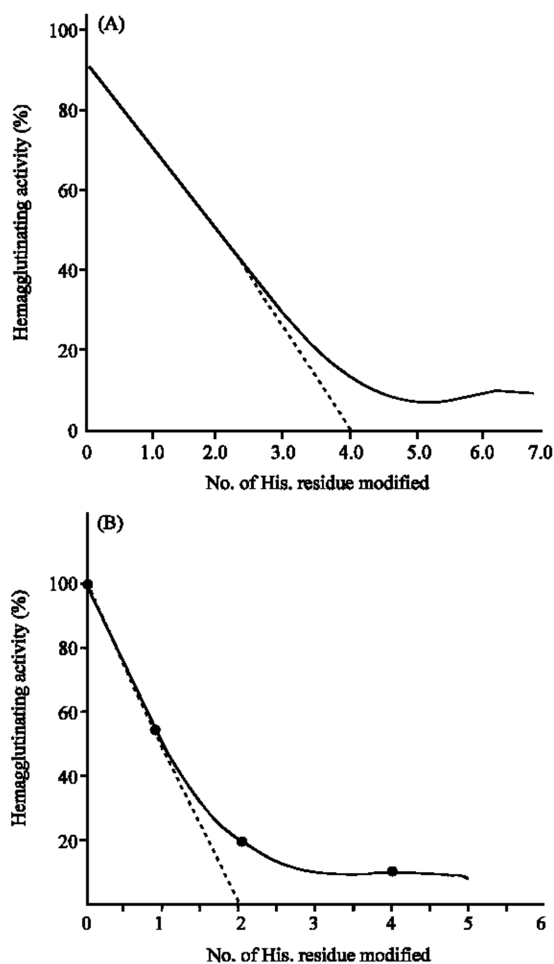

Fig. 4: Correlation of the hemagglutinating activity of MSL-1 (A) and MSL-2 (B) with the modification of histidine residues by DEPC at pH 7.2. Percentage of hemagglutinating activities remaining after modification as compared to that of the native hemagglutinating activities were plotted against the number of residues modified

histidine residues, respectively were modified (Table 5). As shown in Fig. 4A and B the hemagglutinating activities of the lectins decreased markedly during modification with DEPC and less than 20% activities retained after modification of four and two histidine residues per molecules of MSL-1 and MSL-2, respectively.

Table 6: Effect of modification with DTNB on hemagglutinating activities and carbohydrate binding properties of MSL-1 and MSL-2

Proteins	Chemical treatment	No. of residues modified per mole of lectin	Hemagglutination activity (%)	Binding ability to Sepharose 4B (%)
MSL-1	-	0	100	100
MSL-2	-	0	100	100
MSL-1	DTNB	10.2±0.3	5	5
MSL-2	DTNB	4.6±0.3	10	10

Modification of -SH groups

DTNB-5,5′-dithiobis -(2-nitrobenzoic acid) (DTNB) is very specific for chemical modification of -SH groups in protein (Ellman, 1959). As given in Table 6 about ten and five sulfhydryl groups per molecule of MSL-1 and MSL-2, respectively were modified with DTNB and the resulting modified lectins lost their hemagglutinating activities and binding ability to Sepharose 4B almost completely.

DISCUSSION

Chemical modification of active site amino acid side chain will alter their properties and bring about a change in the biological activity of the protein, if they contribute to it. This change in activity can be measured for a variety of modification of different amino acid side chains and this is an idea of those involved in the biological activity can be obtained.

As investigation of this sort on mulberry seed lectins would, it was expected, enable us to compare the amino acid involved in binding with well characterized lectins of similar galactose specificity such as ricin, abrin, castorbean hemagglutinin, abrus bean hemagglutinin, potato lectin, etc.

An extensive acetylation of the lysyl residues of MSL-1 and MSL-2 with acetic anhydride led to a almost complete loss in their hemagglutinating activities and binding abilities to Sepharose 4B. Since acetic anhydride is not specific for lysyl residues but also modified tyrosyl residues. Citraconic anhydride, which shows a preference for the amino groups (Dixon and Perham, 1968; Klotz, 1967) was used to modify the lysyl residues of mulberry seed lectins. Citraconylation of the lectins did not affect their biological activities, indicating that lysyl residues of MSL-1 and MSL-2 are not involved in saccharide binding. It might be concluded from these data tyrosyl groups of MSL-1 and MSL-2 are associated with the lectins activities. This finding was also supported from the observation that more than 75% biological activities of the lectins, were regained after treatment with hydroxyl amine.

Acetylation of MSL-1 and MSL-2 with N-acetyl imidazole which gave acetylation of both lysyl and tyrosine residues and the modified lectins lost their activities completely. The loss in activity of MSL-1 and MSL-2 by acetylation was reversible to 75-80% by treatment with hydroxylamine suggesting also that tyrosyl residues located at or near the saccharide binding site(s). This was further substantiated from the observation that the activities of the lectins were retained when four and two tyrosine residues in MSL-1 and MSL-2, respectively were protected from modification in the presence of specific saccharide 0.2 M galactose. RicinD, like these two lectins required tyrosine residues for its activity (Hatakeyama *et al*., 1986). The data presented here also provide evidence for the noninvolvement of arginine and tryptophan residues in binding saccharides.

Spectroscopic pattern clearly indicated that histidine residues were modified with DEPC and at pH 7.2, eight and six histidine residues per molecule of MSL-1 and MSL-2, respectively were eventually modified. In the hemagglutinating activity test, about 20% activities were formed in the modified MSL-1 and MSL-2, in which four and two histidine residues, respectively were ethoxyformylated (Fig. 4A and B and Table 5). It is note worthy that in the complexes with specific saccharides, 0.2 M galactose, four histidine and two histidine residues per molecule of MSL-1 and MSL-2, respectively were protected from modification with retention of hemagglutinating activities.

These results strongly suggest that the loss of the saccharide binding ability of MSL-1 and MSL-2 on treatment with DEPC are due to modification of histidine residues. Castor bean hemagglutinin, like these lectins, required histidine residues for its activity (Absar *et al*., 1989).

The present data also gave evidence that the thiol groups of MSL-1 and MSL-2 are involved in carbohydrate binding properties of the lectins. Thiol groups are also found to be involved in the saccharide binding property of galactose specific *Pseudomonas aeruginosa* bacterial lecins (Pal *et al*., 1989).

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