http://www.pjbs.org

PJBS

ISSN 1028-8880

# Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 9 (4): 674-680, 2006
ISSN 1028-8880


# Studies on the Chemical Modification of Mulberry Seed Lectin (MSL-1) and its Effect on Hemagglutinating Activity

M.A. Hossain, M.T. Hossain and N. Absar
Department of Biochemistry and Molecular Biology,
University of Rajshahi, Rajshahi-6205, Bangladesh

**Abstract:** The lectin, MSL-1 was subjected to various chemical modifications in order to ascertain the amino acid residues responsible for their hemagglutinating activity. Modification of MSL-1 with acetic anhydride blocked nineteen amino groups and five tyrosine residues per molecule of lectin and decreased complete hemagglutinating activity. De-O-acetylation regenerated four of the tyrosine residues and resulted in a recovery of 80% activity. The presence of inhibitory saccharide galactose, a significant protecting effect was observed and only 1.49 tyrosine residues and fifteen amino groups were found to be modified with significant retention of hemagglutinating activity. The treatment of lectin with citraconic anhydride showed that nineteen amino were modified with the loss of 25-30% hemagglutinating activity. Modification of lectin with N-acetyl imidazole resulted in acetylation of fifteen amino groups and six tyrosine residues per molecule. De-O-actylation also regenerated 4.5 tyrosine residues with the retention of 75% of its hemagglutinating activity. When modification was conducted in the presence of galactose, about 3.5 tyrosine residues were protected from modification with 80% hemagglutinating activity. Successive addition of NBS to MSL-1 solution resulted in the modification of five tryptophan residues per molecule of lectin at pH 6, 5 and 4, respectively with the loss of 20-30% hemagglutinating activity. With DEPC at pH 7.2, eight histidine residues were modified and in the presence of inhibitory saccharide galactose, five histidine residues were protected from modification with the retention of 90% hemagglutinating activity. This was further confirmed from the finding that the activity was regenerated when His-modified MSL-1 was treated with hydroxylamine. The overall modification studies indicated that four tyrosine and five histidine residues were located at the saccharide-binding site of the lectin. However, modification of tryptophan and lysine had no effect on the hemagglutinating activity.

**Key words:** Lectin, (MSL-1) Mulberry Seed Lectin-1, chemical modification, hemagglutinating activity

## INTRODUCTION

Lectins are multivalent carbohydrate binding and cell-agglutinating proteins that have proved to be useful reagents for probing structural features of cell surface glycoproteins and glycopeptides by affinity chromatography (Lis and Sharon, 1973). Lectins are currently attracting much interest primarily because they serve as invaluable tools in diverse area of biomedical research (Hossain *et al*., 2004). The lectins show certain interesting biological properties such as agglutination of tumor cells, blood group specific hemagglutinating activity, mitogenic activity against lymphocytes, precipitation reactions with some species of polysaccharides and glycoproteins etc.

Lectins are similar to enzymes in that they exhibit a specific interaction with particular molecule, but are by definition restricted to interactions with sugars. The carbohydrate binding site(s) of a lectin contain amino acids with side chain that contribute to the association of specific saccharide groups with that lectin, in a manner to an enzyme-substrate association. An enzyme active site differ from a lectin active site as the amino acid side chains, by their interactions with the substrate and other amino acid side chains, bring about the catalytic conversion of the substrate (Ashford *et al*., 1981).

The identification of specific amino acid residues within the active sites of biopolymer is important for understanding the relationship between their structure and biological activity. Chemical modification of proteins serves as a tool to identify the amino acid residues involved in their binding (Horiike and McCormick, 1979). In this paper, we demonstrated chemical modification of various amino acid side chains of Mulberry Seed lectins-1 (MSL-1) that helped to identify the essential amino acid residues at the active site of the lectin.

**Corresponding Author:** Md. Anowar Hossain, Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6205, Bangladesh Tel: +88-0721-750041/4109 Fax:+88-0721-750064

## MATERIALS AND METHODS

Mulberry seeds were collected from the Bangladesh Sericulture Research and Training Institute (BSRTI), Rajshahi, Bangladesh in 1998-99. Sephadex G-150, DEAE-cellulose and Sepharose-4B were purchased from Sigma chemicals, USA. All other reagents were used in analytical grade.

**Extraction and purification crude lectins:** We described the detailed procedure for extraction, purification and characterization of three mulberry seed lectins in our previous paper (Hossain *et al*., 2004)

**Hemagglutinating activity:** The hemagglutination of albino rat red blood cells (erythrocytes) by native and modified MSL-1 was determined at room temperature according to the method (Lin *et al*., 1981). Before assay, all the modified samples were dialyzed against distilled water against 5 mM phosphate buffer saline, pH 7.4.

**Chemical modification of MSL-1:** All treatment were performed under mild conditions and with reagents that had a high specificity for particular amino acid side chains. The reagents were also chosen because the reaction products could be determined quantitatively either directly or indirectly after second treatment. Also where possible, reagents were chosen where the modification produced could be reversed, regenerating the original amino acid residues. Thus, if the lectin lost activity and the modification were reversible and on reversal activity was regained, extra weight could be added to the involvement of that amino acid in the biological activity rather than the effect being due to side reaction.

**Acetylation with acetic anhydride:** The method employed was based on that described by Rice and Etzler (1975). Stirring continuously, 1 μL portion of acetic anhydride was added to 2 mL of MSL-1 (0.6-1.0 mg) in 0.5 mL of 50% saturated sodium acetate buffer, pH 8.3, at 0°C for four times over the course of 1 h. The pH was maintained between 8 and 9 by addition of 0.1 M NaOH. After 1 h, the treated samples were then dialyzed against distilled water followed by dialysis against phosphate buffer saline, pH 7.4 at 4°C.

De-O-acetylation of acetylated lectin sample was made upto 1 M with respect to hydroxylamine by addition of an equal volume of 2 M hydroxylamine-HCl adjusted to pH 7.5 with 0.1 M NaOH. The solutions were mixed and left with occasional stirring for 15 min at 20°C followed by exhaustive dialysis against distilled water at 4°C (Ashford *et al*., 1981). The total number of free amino groups in the native protein was estimated with reference to bovine serum albumin by tri-nitrobenzene sulfonic acid (TNBS) according to the method (Habeeb *et al*., 1967) The number of amino groups modified was determined by estimation of remaining free amino groups with the same reagent.

**Citraconylation with Citrconic anhyride:** The amino groups were also modified with Citraconic anhydride by the method (Habeeb *et al*.,1958). Two microliter of protein solution (0.6-1.0 mg $mL^{-1}$) in 0.5 mL of Tris-HCl buffer, pH 8.4, was treated with approximately 40-fold molar excess of the reagent at 4°C for 1 h. The number of amino group modified was calculated by determination of the unmodified amino groups with TNBS as described by Habeeb *et al*. (1967).

**Acetylation with N-acetylimidazole:** Acetylation with N-acetylimidazole was carried was carried out essential according to the method (Riordan *et al*., 1965). The reaction was followed at 20°C. Two microliter of the lectin (MSL-1) in 0.5 mL of 0.05 M sodium borate buffer at pH 7.5, was mixed approximate with 30 fold molar excess of N-acetylimidazole. The reaction mixture was kept at room temperature for 1 h.

The number of O-acyl group was determined from the change in absorbance accompanying the reaction as described Riordan *et al*. (1965). De-O-acetylation of the acetylated lectin was done with 1 M hydroxylamine-HCl as described (Ashford *et al*., 1981). Before testing the hemagglutinating activity, the treated samples were dialyzed against phosphate buffer saline, pH 7.4 at 4°C for 12 h.

**Modification of tryptophan resiudes with N-bromosuccinimide:** The modification of tryptophan residues with N-bromosiccinimide(NBS) was carried out according to the method (Spande and Witkop, 1967). Oxidation was performed in the following way: An aliquot (2.0 mL) of protein solution ($A_{280}$ = 0.7-1.0) was placed in a quartz cell provided with a miniature-stirring bar and the absorption spectrum was recorded by a Shimadzu double Beam Spectrophotometer (UV-180). The experiment was conducted in an air-conditioned room at 20°C. Then 1 μL of NBS solution ( $2.4\times10^{-3}$ M) was stirred slowly for 2-3 min and absorption spectrum was recorded. To obtain the titration curve for oxidation of tryptophan in lectin, NBS solution was added successively until the decrease in absorbance at 280 nm ceased. The number of tryptophan residues oxidized were calculated according to method (Spande and Witkop, 1967).

**Modification of histidine residues:** Histidine residues of MSL-1 were modified with diethyl pyrocarbonate (DEPC) (Muhlrad *et al*., 1969). The method was essentially the same procedure of Melchior and Fahrney (1970). The reaction was carried out at 20°C in a quartz cell and pursued by difference spectroscopy. A base line was first recorded by lacing protein solution in respective buffer in the reference and sample cell. Two microliter of protein solution (0.6-1.0 mg $mL^{-1}$) in 0.5 mL of 20 mM sodium phosphate buffer, pH 7.2 in the sample cell, was added different amount (5-20 μL) of Diethyl Pyrocarbonate (DEPC) solution. At intervals of time, the difference spectra were recorded against protein solution in the reference cell, which received same quantity of buffer solution instead of DEPC solution. The number of histidine residues modified was calculated from the difference in molar extinction at 240 nm ($\Delta\varepsilon$ = 3,200 $cm^{-1}$ $M^{-1}$). In a different set of experiments, the same amount of lectin was treated with dietyl pyrocarbonate in the presence of galactose.

## RESULTS

**Acetylation of MSL-1 with acetic anhydride:** Acetic anhydride modified lysine including the terminal α-amino and the phenolic hydroxyl group such as tyrosine residue (Dixon and Perham, 1968 ). Acylation of tyrosine phenolic groups can be distinguished from that of amino groups by a decrease in absorption at 278 nm accompanying the former. The effects of acetic anhydride on hemagglutinating activity of MSL-1 are given in Table 1.

On the reaction of MSL-1 with acetic anhydride, Nineteen $NH_2$ groups including the α-amino group as well as five tyrosine residues were modified and resulted completely loss in hemagglutinating activity of the modified protein, suggesting that lysine and/or tyrosine residues were responsible for carbohydrate binding property of MSL-1. De-O-acetylation of the modified protein on treatment with 1 M hydroxylamine at pH 7.5, regenerated about four of the tyrosyl residues and also resulted in the recovery of about 80% of its hemagglutinating activity relative to that of the native lectin.

De-O-acetylation process is known to remove only the O-acyl group of tyrosine but not the N-acyl of amino groups (Simpson *et al*., 1963). Regeneration of lectin activity was therefore due to the availability of phenolic-OH tyrosine groups. Such finding indicated that tyrosine residues of the lectin might be involved in the biological activity of MSL-1. The involvement of such residues in the binding site was also confirmed from the finding that when lectin was modified with acetic anhydride in the presence of inhibitory saccharide, 0.2 M galactose. A significant protective effect was observed and only 1.49 tyrosine residues and fifteen amino groups were found to be modified in MSL-1 with significant retention of hemagglutinating activity.

Table 1: Effects of modification with Acetic anhydride, Citraconic anhydride and N-acetylimidazole on hemagglutinating activity of MSL-1

| Treatment on MSL-1 | Types groups modified | No. of residues modified (mol $mol^{-1}$ protein) | Hemag-glutinating activity (%) |
|---|---|---|---|
| None | - | - | 100 |
| Acetic anhydride | Tyr* | 5.05 | 0.0 |
| | $NH_2$# | 19 | 0.0 |
| Acetic anhydride (In presence of 0.2 M galactose) | Tyr* | 1.49 | 80 |
| | $NH_2$# | 15.3 | 80 |
| Acetic anhydride followed by 1 M hydroxylamine, pH 7.5 | Tyr* | 0.8 | 80 |
| | $NH_2$# | 18.7 | 80 |
| Citraconic anhydride | $NH_2$# | 19.6 | 70 |
| Citraconic anhydride (In presence of 0.2 M galactose) | $NH_2$# | 18.0 | 75 |
| Molar ratio: N-acetylimidazole to protein (30:1) | Tyr* | 5.87 | 0 |
| | $NH_2$# | 15.2 | 0 |
| Acetylation (In presence of 0.2 M galactose) | Tyr* | 2.39 | 80 |
| | $NH_2$# | 12.5 | 80 |
| Acetylation and subsequent Deacetylation by 1 M hydroxylamine, pH 7.5 | Tyr* | 1.5 | 75 |
| | $NH_2$# | 14.8 | 75 |

*No. of residues modified as determined from the change of absorbance of 278 nm according to Riordan *et al*.(1965) Method .
# No. of residues modified as calculated by Habeeb *et al*.(1967) method.

**Citraconylation of MSL-1:** Treatment of lectin with citraconic anhydride results in the binding of citrconyl groups to free amino groups (Habeeb *et al*., 1958). The effect of citraconylation on hemagglutinating activity of MSL-1 is shown in the Table 1. It is clear that citraconylation of MSL-1 reduced moderately its ability to induce indirect hemagglutination of rat red blood cells. The nineteen of the available amino groups of MSL-1 was found to be modified after treatment with citraconic anhydride and the hemagglutinating activity of modified MSl-1 was lost about 25-30% relative to that of the native one.

**Acetylation of the MSL-1 with N-acetylimidazole:** N-acetylimidazole reacts with both amino and tyrosyl group, but is more selective for tyrosine under mild conditions. For example, it was found to be selectively acetylated six tyrosine groups in bovine carboxypeptidase without affecting amino groups (Simpson *et al*., 1963). The decease in the absorbance at 278 nm, i.e. $\Delta\varepsilon_{278}$ nm = 1160 $mol^{-1}$, on O-acetylation of N-acetyltyrosine has been

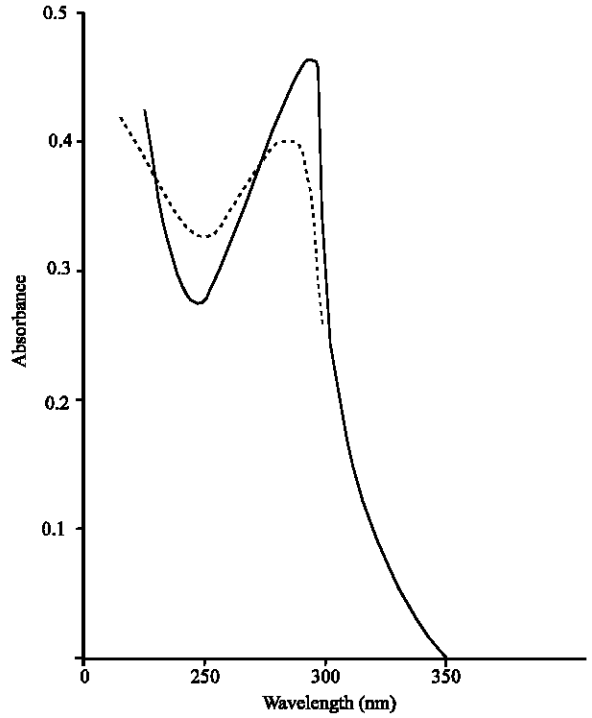


Fig. 1: Acetylation of tyrosyl residues in MSL-1 with N-acetylimidazole. Acetylations were carried out with 30-fold excess of the reagent as described in the Materials and methods. ( ___ ) spectrum of the native protein, (-----) spectrum of acetylated protein.

used to determine the number of tyrosine residues modified in each protein (Riordan *et al*., 1965). The UV-absorption spectra of native and N-acetylimidazole modified MSL-1 are shown in Fig. 1. As observed, the absorption of acetylated MSL-1 decreased around 278 nm. The treatment of MSL-1 with N-acetylimidazole resulted in acetylation of about fifteen amino groups and six tyrosine residues per molecule, and the hemagglutinating activity of the modified MSL-1 was lost completely (Table 1). Deacetylation with 1M hydroxylamine, pH 7.5 regenerated 4.5 tyrosine residues and lectin retained 75% of its hemagglutinating activity. Again, when the modification reaction was conducted in the presence of galactose, about 3.5 tyrosine residues were found to be protected from modification with retention of 80% hemagglutinating activity relative to that of native MSl-1.

**Modification of tryptophan residues with N-bromosuccinamide:** Successive addition of NBS to MSL-1 solutions resulted a decrease in the absorbance at 280 nm due to the transformation of tryptophan to oxindole, a much weaker chromophore at this wavelength

Table 2: Effects of modification with N-bromosuccinamide (NBS) and Diethyl Pyrocarbonate (DEPC) on hemagglutinating activity of MSL-1

| Modifying condition | Types of residues modified | No. of Trp residues modified (mol $mol^{-1}$ protein) | Hemag-glutinating activity (%) |
|---|---|---|---|
| Native MSL-1 | - | 0.0 | 100 |
| MSL-1 +NBS PH 6 | Trp | 3.5 | 80 |
| MSL-1+NBS PH 5 | Trp | 4.1 | 75 |
| MSL-1+NBS PH 4 | Trp | 4.9 | 70 |
| MSL-1+NBS (In presence of 0.2M galactose) pH 4 | Trp | 4.75 | 70 |
| MSL-1+DEPC | His | 8.25 | 0 |
| MSL-1+DEPC( In presence of 0.2 M galactose) | His | 3.2 | 90 |
| DEPC modified MSL-1 following 0.2 M hydroxylamine, pH 7.2 | His | 0.84 | 95 |

(Patchornik *et al*., 1958). At pH 4.0, five tryptophan residues in MSL-1 were modified with N-bromosuccinamide and the modified MSL-1 lost only about 30% activity. Again, no protection effect was observed when the modification was performed in the presence of 0.2 M galactose, an inhibitory saccharide. The titration curve of NBS oxidation of tryptophan in MSL-1 is shown in Fig. 2. As observed the absorbance at 280 nm decreased while that around 260 nm increased with the addition of higher amount of NBS solution. It was also found that the extent of tryptophan modification with NBS was influenced slightly by changes of pH. For example, three-tryptophan residues $mol^{-1}$ were oxidized at pH 6.0 where as four and five tryptophan residues $mol^{-1}$ were oxidized at pH 5 and 4, respectively. Effects of tryptophan modification on hemagglutinating activity of MSL-1 are shown in Table 2.

**Modification of histidine residues with DEPC:** The reactivity of DEPC has been directed towards the preferential modification of histidine residues in protein (Miles, 1977; Muhlrad *et al*., 1969). As seen in the Fig. 3, the reaction proceeded with increase in the absorption at 240 nm, which is characteristic of carboethoylation of imidazole ring of histidine residues (Ovadi *et al*., 1967). Neither tryptophan nor tyrosine residues were modified through this modification since no noticeable change was observed around 280-300 nm region in the difference spectra during the reaction.

Again, it was fond that nearly 75% modification reaction completed within 19 min; and afterwards the reaction proceeded slowly and the modification reaction reached a plateau after 27 min. With DEPC at pH 7.2, eight histidine residues per molecule were modified, but in the presence galactose, a potent inhibitor for MSL-1, only 3.2 histidine residues per molecule were modified, indicating

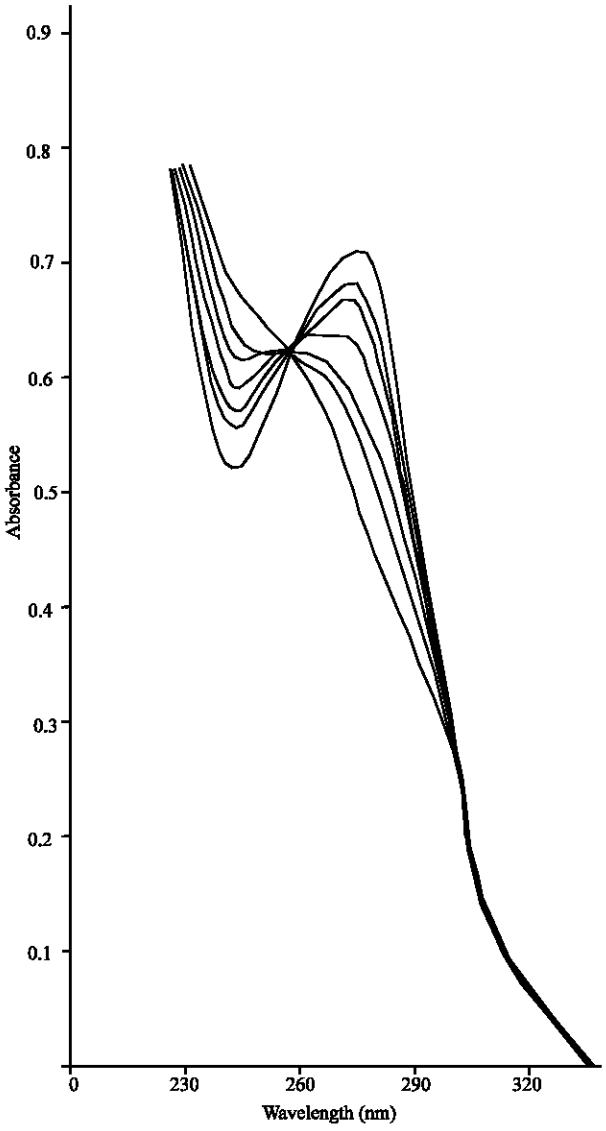


Fig. 2: NBS oxidation of MSl-1 at pH 4. The concentration of MSL-1 was $8.3\times10^{-6}$ M in 0.1 M buffer of respective pH. The reaction was followed at 20°C

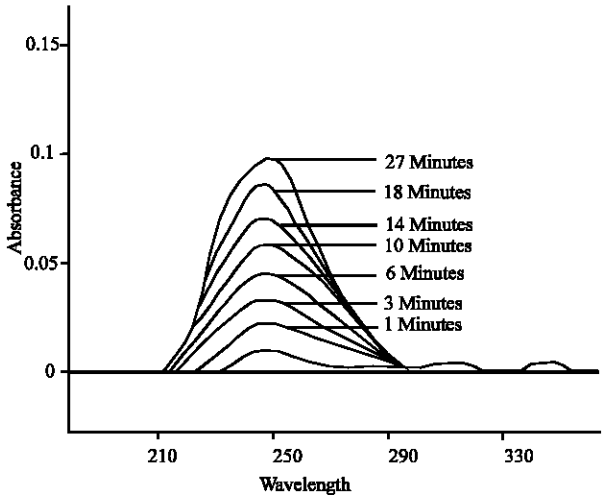


Fig. 3: Ultraviolet difference absorption spectra of DEPC modified MSL-1. The experiment was performed at 20°C. Solution of MSL-1 ($8.3\times10^{-6}$ M) in 20 mM sodium phosphate buffer, pH 7.2, was placed in the reference and sample cuvette. The spectra were run at different time interval after addition of 5 μL DEPC solution to the sample cuvette. The reference cell contained all ingredients except DEPC but received equal amount of buffer

that five-histidine residues/mol. were protected from this modification reaction in the MSL-1-complexes. The effects of modification on the hemagglutinating activity of MSL-1 is presented in Table 2.

The hemagglutinating activity of MSL-1 towards rat red blood cells was lost completely after modification of eight histidine residues with DEPC. However, when modification reaction was done in presence of galactose, the resulting derivative of MSL-1 containing five-ethoxyformylated histidine residues/mol retained 90% of the hemagglutinating activity. This suggested that Histidine residues in MSL-1 were associated with activity. This was further confirmed from the finding that the activity was regenerated when His-modified MSL-1 was treated with hydroxylamine.

## DISCUSSION

Proteins in a solution exist in various conformations. In case of an enzyme or a lectin the different conformers may be associated with various degrees of activity and some conformers may be inactive. If substitution or other modifications of amino acid residues destroy lectin activity, the most obvious explanation of such a finding is that the resides are part of the active site and involve in the binding of the ligand. It is however, possible that the residues in question are not a part of the active site but are required for the maintenance of the active conformation.

An extensive acetylation of the lysyl and tyrosine groups of MSL-1 with acetic anhydride led to total loss of its hemagglutinating activity. When the acetylation reaction was carried out in the presence of galactose four tyrosine residues, four amino groups of MSL-1 were protected from modification, and the modified lectin retained 80% of its hemagglutinating activity. These results suggested that tyrosyl/amino groups of MSL-1

were involved in hemagglutinating activity. The involvement of tyrosine residues in hemagglutinating activity was confirmed from the finding that the hemagglutinating activity of acetic anhydride treated MSL-1 could be reversed more than 80% by subsequent treatment with hydroxylamine.

Since acetic anhydride is not specific for amino groups, but also modifies tyrosine residues. Citraconic anhydride that shows preference for the amino groups was used to modify the amino groups of MSL-1 (Dixon and Perham, 1968; Gunther *et al*., 1973). Citraconylation of MSL-1 caused mild loss of its hemagglutinating activity suggested that amino groups might not be involved in the saccharide binding of the lectin.

The involvement of tyrosine residues in the saccharide binding ability of MSL-1 was further substantiated by the results obtained from the acetylation of MSL-1 with N-acetylimidazole. Acetylation by N-acetylimidazole led to complete loss of the hemagglutinating activity of MSL-1 and the loss in activity of acetylated MSL-1 was reversed by treatment with hydroxylamine. From these finding it can be concluded that tyrosine residues are important for hemagglutinating activity of MSL-1.

The residual hemagglutinating activity of modified MSL-1, in which about three tryptophan residues/moles oxidized at pH 6.0, was 80%, but the subsequent loss of the residual activity after oxidation of additional two tryptophan residues /mole at pH 4.0 was only further 10%. It may be suggested from the results that tryptophan residues in MSL-1 were not located at the saccharide-binding site, and the moderate decrease in hemagglutinating activity with the modification of tryptophan residues might be due to the conformational change of the protein at lower acidic pH or due to the change in microenvironment of tryptophan residues with pH changes.

Spectrophotometric data clearly indicate that only histidine residues in MSL-1 was modified with DEPC at pH 7.2 (Fig. 3). The hemagglutinating activity of MSl-1 was destroyed completely after modification it with DEPC. It is noteworthy that in the presence of galactose, five histidine residues per molecule were protected from the modification with the retention of about 90% of its hemagglutinating activity.

Furthermore, It was found that a marked decrease in activity resulted from ethoxyformylation of MSL-1 with DEPC, which could be reversed by treatment of the modified lectin with 0.2 M hydroxylamine, at pH 7.2. From these results it may be suggested that histidine residues in MSL-1 are essential for hemagglutinating activity.

## ACKNOWLEDGMENTS

We are grateful to Dr. Abdul Aziz Sarkar, senior scientific officer, Bangladesh Sericulture Research and Training Institute (BSRTI), Rajshahi, Bangladesh for supplying mulberry seeds during our research works.

## REFERENCES

Ashford, D., R. Menon, A. K. Allen, and A. Neuberger, 1981. Studies on the chemical modification of potato (Solanum tuberosum) lectin and its effect on haemagglutinating activity. Biochem. J., 199: 399-408.

Dixon, H.B.F. and R.N. Perham, 1968. Reversible blocking of amino groups with citraconic anhydride. Biochem. J., 109: 312-314

Gunther, G.R., J.L. Wang, I. Yahara, B.A. Cunningham and G.M. Edelman, 1973. Concanavalin a derivatives with altered biological activities. Proc. Nat. Acad. Sci., USA, 70: 1021-1026.

Habeeb, A.F.S.A., H.G. Cassidy and S.J. Singer, 1958. Molecular structural effects produced in proteins by reaction with succinic anhydride. Biochim. Biophys. Acta, 29: 587-593.

Habeeb, A.F.S.A., 1967. Preparation of enzymically active, water-insoluble derivatives of trypsin. Arch. Biochem. Biophys., 119: 264-268.

Horiike, K. and B.K. McCormick, 1979. Chemical modification of proteins J. Theor. Biol., 79: 403-414

Hossain, M.A., S.M. R.Islam and N. Absar, 2004. Purification and Characterization of Lectins from Mulberry Seeds (*Morus alba* L.). Pak. J. Biol. Sci., 7: 1805-1813.

Lis, H. and N. Sharon, 1973. The biochemistry of plant lectins (phytohemagglutinins). Annu. Rev. Biochem., 42: 541-574.

Lin, J.Y., T.C. Lee, S.T. Hu and T.C. Tung, 1981. Isolation of four isotoxic proteins and one agglutinin from jequiriti bean (*Abrus precatorius*). Toxicology, 19: 41-51.

Muhlrad, A., G. Hegy and M. Horanyi, 1969. Studies on the properties of chemically modified actin. 3. Carbethoxylation. Biochem. Biophys. Acta, 181: 184-90.

Melchior, Jr. W.B. and D. Fahrney, 1970. Ethoxyformylation of proteins. Reaction of ethoxyformic anhydride with alpha-chymotrypsin, pepsin and pancreatic ribonuclease at pH 4. Biochemistry, 9: 251-258

Miles, E.W., 1977. Modification of histidyl residues in proteins by diethylpyrocarbonate. Methods Enzymol., 47: 431-442.

Ovadi, J., S. Libor and P. Elodi, 1967. Spectrophotometric determination of histidine in proteins with diethylpyrocarbonate. Acta. Biochem. Biophys., 2: 455-458.

Patchornik, A., W.B. Lawson and B. Witkop, 1958. Selective cleavage of peptide bonds. II. The tryptophyl peptide bond and the cleavage of glucagon. J. Am. Chem. Soc., 80: 474.

Riordan, J.F., W.E.C. Wacker and B.L. Vallee, 1965. N-Acetylimidazole: A reagent for determination of 'free' tyrosyl residues of proteins. Biochemistry, 4: 1758-1765

Rice, R.H and M.E. Etzler, 1975. Chemical modification and hybridization of wheat germ agglutinins. Biochemistry, 14: 4093-4099.

Simpson, R.T., J.F. Riordan and B.L. Vallee, 1963. Functional tyrosyl residues in the active center of bovine pancreatic carboxypeptidase-A. Biochemistry, 2: 616-622.

Spande, T.F. and B. Witkop, 1967. Determination of the tryptophan content of proteins with N-bromosuccinimide. Methods Enzymol., 11: 498- 506.