CHAPTER 3

Chemical modification studies on lectins from *Agrobacterium radiobacter*

SUMMARY

Agrobacterium radiobacter produces two lectins (Lectin I and Lectin II). Incubation of Lectin I with N -bromosuccinimide (NBS) resulted in 87 % loss of haemagglutination activity with concomitant modification of two tryptophan residues. Treatment with Woodward's reagent K resulted in 60 % loss of activity. Lectin II was totally inactivated with Diethylpyrocarbonate (DEP) whereas N-bromosuccinimide (NBS) brought about only 50 % loss of the lectin's haemagglutination activity. Lectin II was also inactivated by carboxylate groups modifying reagent, Woodward's reagent K. The loss in activity was 75 %. The results indicate that carboxyl side chains of acidic amino acids and the indole side chain of tryptophan play a role in the saccharide binding activity of Lectin I. However, for Lectin II, histidine, carboxyl groups of acidic amino acids and to a lesser extent tryptophan have a role in it's saccharide binding activity.

3.1 INTRODUCTION

Identification of specific amino acid residues within the active site of a biologically active proteins is important for understanding the relationship between its structure and function as well as the role played by specific amino acid side chains in its biological activity. Chemical modification is among the methods used for identifying at least some of the essential amino acid residues involved in substrate binding. Microbial lectins, in general, have been rarely studied with regards to the amino acid residues involved in their sugar binding activities.

Agrobacterium radiobacter produced two types of lectins, Lectin I and Lectin II. Lectin I is a monomer of M_r 37000 and is specific for oligosaccharide glcNAc-glcNAc-man5. Lectin II is also a monomer of M_r 40000 and it is specific for D glucosamine and complex carbohydrates of plant origin. To identify the residues involved in the sugar binding of Lectin I and Lectin II, they were subjected to modification by various chemical modification reagents. The results of this preliminary study are reported in this chapter.

3.2 MATERIALS AND METHODS

Materials

2,4,6 -trinitrobenzenesulphonic acid (TNBS), diethylpyrocarbonate (DEP), 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB), N -acetyl imidazole, imidazole, Woodward's reagent K, phenylglyoxal and N-bromosuccinimide were purchased from Sigma Chemical Company, U. S. A. All other chemicals used

were of analytical grade.

Methods

Chemical modification reactions were carried out using different chemical reagents under their respective reaction conditions. The residual activity of the lectins (Lectin I and Lectin II) was checked in the following manner : a) after modification, the reaction mixtures were dialyzed thoroughly b) lyophilized to dryness c) dissolved in 100 µl of buffer (Tris-HCl 20 mM, pH 7.2) and haemagglutination activity was checked. The HA activity was determined as described in chapter 2 section 2.2.12.

3.2.1 Reaction with N -bromosuccinimide

This was carried out by incubating both the lectins (250 µg) with different concentrations of NBS at pH 4.5 (0.1M acetate buffer). The number of tryptophan residues getting modified were determined spectrophotometrically by monitoring the decrease at A_{280} after each addition of a small aliquot of 1 mM NBS (1). The residual activities after modification of tryptophan residues, in the protein samples, were monitored by haemagglutination.

3.2.2 Reaction with 2,4,6-trinitrobenzenesulphonic acid (TNBS)

Both the lectins (250 µg) were incubated seperately with varying concentrations of TNBS (0.1 - 1 mM) in 4 % (w/v) sodium bicarbonate at 37°C in the dark. The reaction mixture was dialyzed thoroughly against Tris buffer (20 mM, pH 7.2)

till the pH of the reaction mixture became 7.2. Protein sample incubated with sodium bicarbonate in absence of TNBS served as control (2). The residual activities after modification of lysine residues in the protein were checked by haemagglutination.

3.2.3 Reaction with phenylglyoxal

For modification of arginine residues, 250 µg of both the lectins were incubated with 10 % (w/v) phenylglyoxal in methanol at pH 8.0 and at 30°C for 30 min. Lectin samples in same quantity of methanol without phenylglyoxal, served as control. The residual activities of the lectins were checked after extensive dialysis (3).

3.2.4 Reaction with diethylpyrocarbonate (DEP)

The reaction was carried out by incubating 250 µg of each lectin at pH 7.2 and 30°C for 30 min., with various con centrations of DEP, freshly diluted with absolute ethanol. The concentration of DEP was determined (4,5) using imidazole buffer. Samples incubated under similar conditions except DEP reagent served as control.

3.2.5 Reaction with N - acetyl imidazole

250 µg of both the proteins (Lectin I and Lectin II) at pH 7.5 were incubated with 10 mM N-acetyl-imidazole for 30 min. at room temperature followed by estimation of residual lectin activities. Proteins incubated in the absence of the reagent were taken as control. (6).

3.2.6 Reaction with 5,5'dithiobis 2-nitrobenzoic acid (DTNB)

The proteins were incubated with 10 mM DTNB at room temperature for 30 min. and the residual activities were checked after dialysis. Lectins incubated in absence of DTNB served as control.

3.2.7 Reaction with Woodward's reagent K

The reaction was carried out by incubating 250 µg of each of the lectins at pH 7.2 at 25°C for 30 min., with various concentrations of WRK (upto 30 mM). The residual activities were checked after dialysis. Protein samples incubated in absence of WRK served as control (7).

3.2.8 Determination of protein concentration of Lectins

The protein concentration of Lectin I and Lectin II were determined by the method of Lowry *et al* (8) by using bovine serum albumin as standard.

3.3 RESULTS

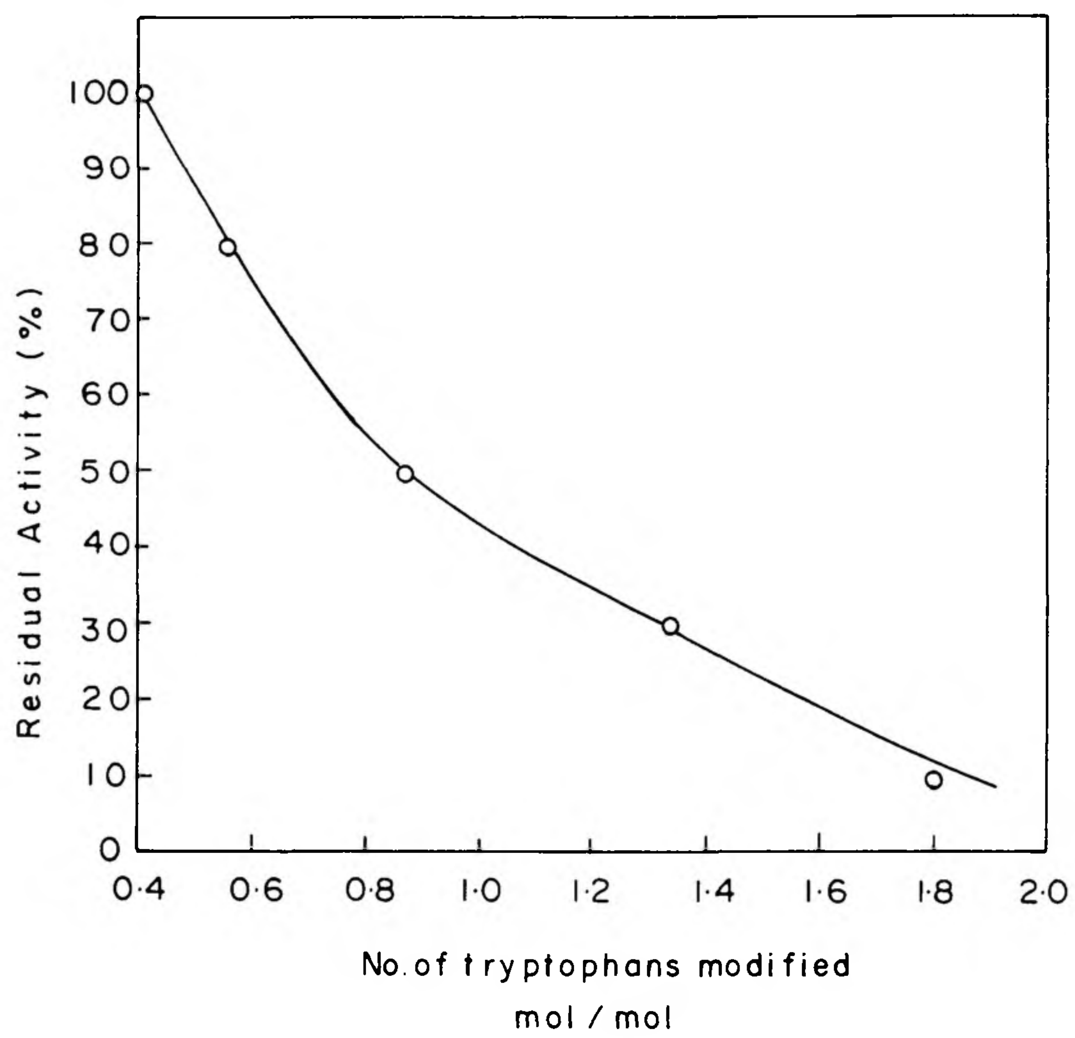
3.3.1 Effect of modification of tryptophan residues

Agrobacterium radiobacter Lectin I lost 87 % of its haemagglutination activity when incubated with NBS at a concentration of 28 µM (Table 3.1). A Total of two tryptophan residues were modified. It can be seen from Figure 3.1 and 3.2 that the lectin lost 50 % of it's activity, when the first tryptophan was modified at 15 µM concentration of NBS. Rest of the activity was lost with the modification of the second tryptophan residue.*Agrobacterium radiobacter*

Table 3.1 : Chemical modification of Lectin I

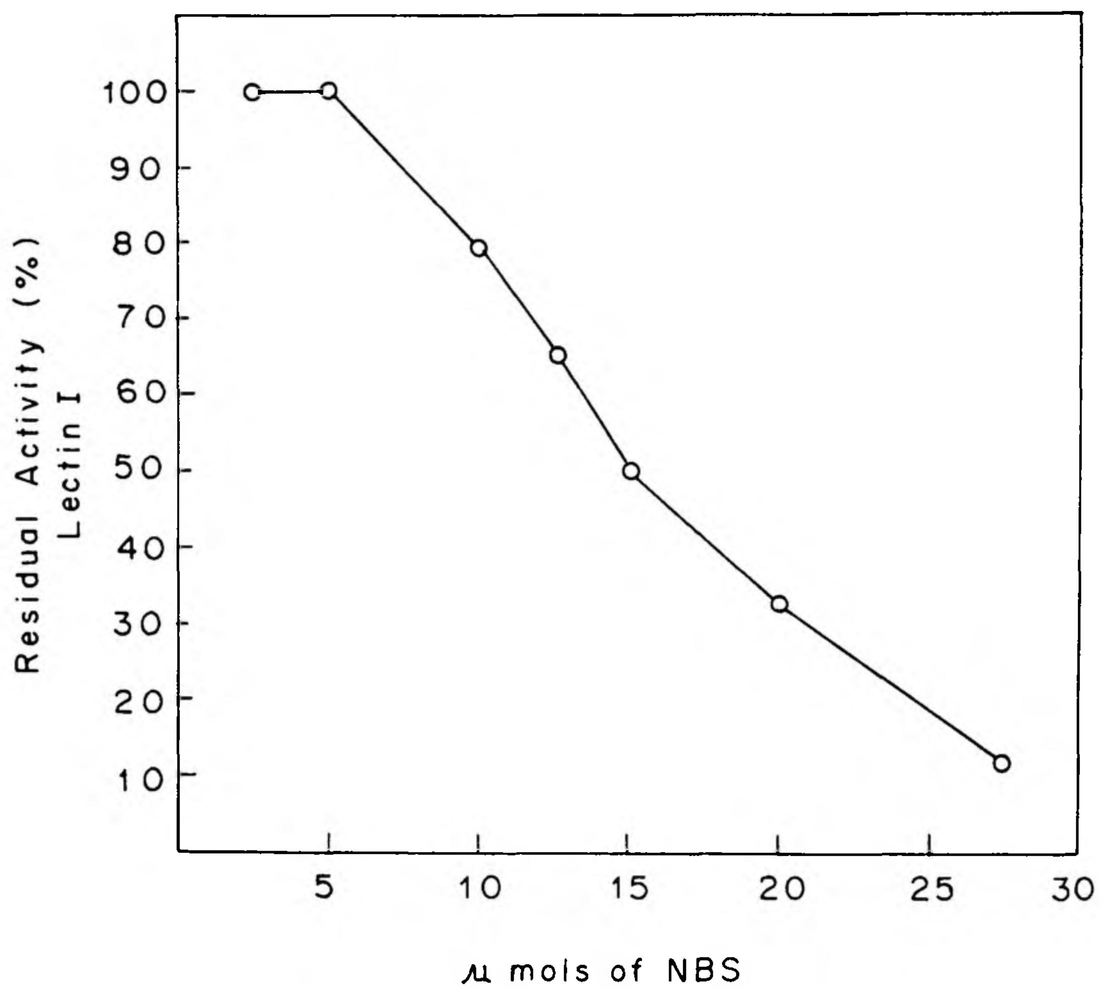
Chemical Treatment	Residues modified	% Residual Activity
None	--	100
N-bromosuccinimide	Tryptophan	12
p-nitrohenylglyoxal	Arginine	100
N-acetyl imidazole	Tyrosine	100
Diethylpyrocarbonate	Histidine	100
Woodward's reagent K	Carboxyl	40
TNBS	Lysine	100
DTNB	Cysteine	100

Figure 3.1 : Loss of activity with modification of tryptophan of Lectin I with N-bromo succinimide



Residual Activity (%)
100
90
80
70
60
50
40
30
20
10
0
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
No. of tryptophans modified
mol / mol

Figure 3.2 : Concentration dependent loss of HA activity of Lectin I with N-bromosuccinimide



Residual Activity (%)
Lectin I
100
90
80
70
60
50
40
30
20
10
5
10
15
20
25
30
μ mols of NBS

Table 3.2 : Chemical modification of Lectin II

Chemical Treatment	Residues modified	% Residual Activity
None	--	100
N-bromosuccinimide	Tryptophan	50
p-nitrohenylglyoxal	Arginine	100
N-acetyl imidazole	Tyrosine	100
Diethylpyrocarbonate	Histidine	0
Woodward's reagent K	Carboxyl	25
TNBS	Lysine	100
DTNB	Cysteine	100

Lectin II lost only 50 % of it's haemagglutination activity with the modification of the only tryptophan present in the lectin. The concentration of NBS required for modification was 20 µM.

3.3.2 Effect of modification of histidine residues

There was no activity loss when Lectin I was treated with Diethylpyrocarbonate (DEP), even when excess reagent was used. Lectin II lost all of it's activity when incubated with the same reagent at 100 µM concentration. At 80 µM concentration of DEP, 50 % loss of activity was observed for Lectin II. (Table 3.2).

3.3.3 Effect of carboxyl groups modification

Carboxyl groups were modified with the help of Woodward's reagent K. Lectin I, when incubated with the reagent, lost 60 % of it's haemagglutination activity. When Lectin II was subjected to Woodward's reagent K modification, it lost almost 75 % of it's haemagglutination activity (Table 3.1, 3.2).

No loss of activity was observed with modification of arginine,lysine,tyrosine or cysteine residues of both of the lectins.

3.4 DISCUSSION

Agrobacterium radiobacter Lectin I is produced extracellularly. This protein is strongly associated with the polysaccharide (EPS) produced by the microorganism. The

lectin was purified by three successive chromatographies and the amount of polysaccharide associated with the lectin decreased gradually with each step. Although after the 2nd step, i.e. hydrophobic chromatography, the sample was pure proteinwise, it had about 10 % of polysaccharide contamination. Only the last step, however, could remove all the polysaccharide associated with it successfully. Therefore the hydroxyapatite chromatography was a very essential step especially for chemical modification studies, as the polysaccharide could have protected the protein from the amino acid modifying chemical reagents.

Lectin I has more acidic amino acid residues. It lost 60 % of it's haemagglutination activity when subjected to carboxyl groups modification. Lectin II however, lost 75 % of it's haemagglutination activity when subjected to Woodward's Reagent K modification. This shows that carboxyl groups in Lectin I and Lectin II contribute in saccharide binding. Hirabayashi *et al.* (9) studied the residues involved in carbohydrate binding of ß-gal specific human lectin by site directed mutagenesis. They suggested that the lectin in which cysteine and tryptophan were reported in the sugar binding site, are actually involved in the stabilization of the binding site and the conservative hydrophilic residues, (Asn^{46} and Glu^{71}) are involved in the saccharide binding. In *Saccharomyces cerevisiae* lectin also, hydrophilic amino acid residues like Asn and Glu have been reported to be important in sugar binding (10). The structural basis for selective sugar recognition by lectins has been investigated by X-ray

crystallography of several plant and animal lectins (11) and it has been shown that the selectivity in the lectin sugar interactions is achieved mainly through hydrogen bonding. Hydrophilic residues play important roles in the function of sugar binding because such residues can readily form hydrogen bonds with hydroxyl groups of specific carbohydrate chains directly or indirectly via a water molecule (12,13).

Lectin I has two tryptophan residues and both of them get modified when subjected to NBS treatment with almost complete loss (87 %) of HA activity. The study clearly indicates that both the tryptophans are present at the saccharide binding site of Lectin I and contribute to sugar binding. In case of plant lectin Ricin D as well as in Abrus lectin, tryptophan has been shown to be involved in sugar binding (7,8). In case of other plant lectins like Concanavalin A, Wheat germ agglutinin, *Momordica charantia* lectin and *Pisum sativum* lectin, a tryptophan residue has been shown to be involved in the carbohydrate binding (14,15,16,17). In case of human lectin (18) as well as in mushroom lectin (19) also, tryptophan has been reported to be involved in sugar binding. Lectin II, however, lost only 50 % of it's haemagglutination activity with the modification of the one and only tryptophan residue. It has a histidine in saccharide binding site, as treatment with (DEP) (100 μM) caused total loss of activity. Basu *et al* (20) have shown histidine in sugar binding site of snail haemolymph lectin.

Modification of arginyl, lysyl, cysteinyl and tyrosyl

residues did not lead to any loss in the sugar binding and haemagglutinating activity of both the lectins, ruling out the possibility of involvement of these residues in sugar binding.

REFERENCES

1. T. F. Spande and B. Witkop (1967) Methods Enzymol. **11** : 498 - 506.
2. A. F. S. A. Habeeb (1966) Anal. Biochem. **14** : 328 - 336.
3. R. B. Yamasaki, D. A. Shimer and R. E. Feeney (1981) Anal. Biochem. **111** : 220 - 226.
4. W. B. Melchior Jr. and D. Fahrney (1970) Biochemistry 9 : 251 - 258.
5. G. E. Means and R. E. Feeney (1971) in "Chemical modification of Proteins" (G. E. Means and R. E. Feeney eds.) Holden-Day Inc. San Francisco pp 214 - 230.
6. V. Sinha and J. M. Brewer (1985) Anal. Biochem. **151** : 327 - 333.
7. T. Hatakeyama, N. Yamasaki and G. Funatsu (1986) J. Biochem. **99** : 1049 - 1056.
8. S. R. Patanjali, M. J. Swamy, V. Anantharam, M. I. Khan and A. Surolia (1984) Biochem J. **217** : 773 - 781.
9. J. Hirabayashi and K. Kasai (1991) J. Biol. Chem. **266** : 23648 - 23653.
10. M. Kundu, J. Basu, A. Ghosh and P. Chakrabarti (1987) Biochem. J. **244** : 579 - 584.
11. W. I. Weis and K. Drickamer (1996) Annu. Rev. Biochem. **65** : 441 - 473.
12. F. A. Quiocho (1986) Annu. Rev. Biochem. **55** : 287 - 315.
13. W. Montfort, I. E. Villafranca, A. F. Monzingo, S. R. Ernst,

B. Katzin, E. Rutenber, N. H. Xuong, R. Hamlin and J. D. Robertus (1987) J. Biol. Chem. **262** : 5398 - 5403.

14. G. S. Hassing and I. J. Goldstein (1971) Biochim. Biophys. Acta **243** : 90 - 97.
15. J. P. Privat, R. Lotan, P. Bouchard, N. Sharon and M. Monsigny (1976) Eur. J. Biochem. **68** : 563 - 572.
16. T. Mazumder, N. Gaur and A. Surolia (1981) Eur. J. Biochem. **113** : 463 - 470.
17. M. Cermakova, G. Entlicher and J. Kocurek (1976) Biochim Biophys Acta **420** : 236 - 245.
18. B. Kohnke-Godt and H. J. Gabius (1991) Biochemistry **30** : 55 - 65.
19. H. Kawagishi and H. Mori (1991) Biochim Biophys Acta **1076** : 179 - 186.
20. S. Basu, C. Mandal and A. K. Allen (1988) Biochem J. **254** : 195 - 202.