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Chemical-modification studies of a unique sialic acid-binding lectin from the snail *Achatina fulica*

Involvement of tryptophan and histidine residues in biological activity

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A unique sialic acid-binding lectin, achatinin$_H$ (ATN_H) was purified in single step from the haemolymph of the snail *Achatina fulica* by affinity chromatography on sheep submaxillary-gland mucin coupled to Sepharose 4B. The homogeneity was checked by alkaline gel electrophoresis, immunodiffusion and immunoelectrophoresis. Amino acid analysis showed that the lectin has a fairly high content of acidic amino acid residues (22% of the total). About 1.3% of the residues are half-cystine. The glycoprotein contains 21% carbohydrate. The unusually high content of xylose (6%) and fucose (2.7%) in this snail lectin is quite interesting. The protein was subjected to various chemical modifications in order to detect the amino acid residues and carbohydrate residues present in its binding sites. Modification of tyrosine and arginine residues did not affect the binding activity of ATN_H; however, modification of tryptophan and histidine residues led to a complete loss of its biological activity. A marked decrease in the fluorescence emission was found as the tryptophan residues of ATN_H were modified. The c.d. data showed the presence of an identical type of conformation in the native and modified agglutinin. The modification of lysine and carboxy residues partially diminished the biological activity. The activity was completely lost after a β-elimination reaction, indicating that the sugars are *O*-glycosidically linked to the glycoprotein's protein moiety. This result confirms that the carbohydrate moiety also plays an important role in the agglutination property of this lectin.

INTRODUCTION

It is well known that the sialoglycoconjugates play important roles in the physiology of normal, transformed and developing cells. A specific approach for the detection of sialic acid residues is the use of sialic acid-specific lectins found in a variety of invertebrates (Marchalonis & Edelman, 1968; Roche & Monsigny, 1979) and in the serum (Tsai *et al.*, 1977). Up until now very few sialic acid-binding lectins have been reported (Marchalonis & Edelman, 1968; Bishayee & Dorai, 1980; Miller *et al.*, 1982; Chowdhury *et al.*, 1985; Ravindranath *et al.*, 1985; Hall & Rowlands, 1974), among which only one is commercially available and some are known to be multispecific (Dorai *et al.*, 1982; Marchalonis & Edelman, 1968; Hall & Rowlands, 1974). To the best of our knowledge the present paper is the first report of an extensive study on the chemical modification of a sialic acid-binding lectin.

Recently we have purified and partially characterized a novel sialic acid-binding lectin, achatinin$_H$ (ATN_H) from the haemolymph of the snail *Achatina fulica* (Basu *et al.*, 1986). It has been recently demonstrated that ATN_H exhibits a remarkable specificity for 9-*O*-acetylated sialic acid, in contrast with the lectins from other invertebrates (Mandal & Basu, 1987). This lectin (ATN_H) therefore differs from all other sialic acid-binding lectins reported so far. We have also demonstrated that ATN_H is highly mitogenic to rat and human lymphocytes at very low doses (Mandal & Chowdhury, 1987).

However, very little information is so far available on the structural features essential for its biological activity. The main objective in undertaking the present investigation was to study the detailed structure–activity relationship in this protein.

In the present study ATN_H was purified by a new simple method. The composition of amino acids and carbohydrate residues has been analysed, and detailed studies regarding the chemical modification of various amino acid residues and carbohydrate residues have been carried out. Such studies indicate the essential involvement of tryptophan and histidine residues in the structural, biological and immunological activities of ATN_H.

MATERIALS AND METHODS

Materials

Snails (*Achatina fulica*) were obtained from local markets in Calcutta. Sepharose 4B was the product of Pharmacia Chemicals, Uppsala, Sweden. All the reagents used were of analytical grade. Sodium borohydride, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide, *N*-bromosuccinimide (NBS), *N*-acetylimidazole,

Abbreviations used: ATN_H, achatinin$_H$; NBS, *N*-bromosuccinimide; SSM, sheep submaxillary (-gland) mucin; Me_3Si, trimethylsilyl; HA, haemagglutinating.

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cyclohexane-1,2-dione, diethyl pyrocarbonate, 2-hydroxy-5-nitrobenzyl bromide, citraconic anhydride, phenyl glyoxal and bovine serum albumin were obtained from Sigma Chemical Co. Glycine methyl ester was prepared by the method of Boissonnas *et al.* (1955).

Purification of ATN_H

ATN_H was purified from the haemolymph of *Achatina fulica* as described by Basu *et al.* (1986), with a little modification. Briefly, the specific protein was passed through a sheep submaxillary-gland mucin (SSM)–Sepharose 4B column, equilibrated with 50 mM-Tris/HCl buffer, pH 8.2, containing 150 mM-NaCl and 30 mM-Ca^{2+} (buffer A). After non-specific washing, the specific protein was eluted out with 50 mM-Tris/HCl buffer, pH 8.2, containing 150 mM-NaCl at 30 °C. The eluted material was found to be homogeneous on alkaline gel electrophoresis (Davis, 1964), SDS/10%-(w/v)-polyacrylamide gel electrophoresis (Basu *et al.*, 1986) and immunodiffusion (Ouchterlony, 1948).

Antiserum

Rabbits were immunized by intracutaneous injection of crude haemolymph (1 mg) without adjuvants three times during 1 week, followed by four subcutaneous injections of the crude haemolymph with complete Freund's adjuvant each at 1 week intervals. Finally a booster injection was given with incomplete Freund's adjuvant after 10 days. Immunosera were collected 2 or 3 days after the last injection.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Immunodiffusion

Immunodiffusion was carried out by the method of Ouchterlony (1948), using 1.5% agarose in 50 mM-Tris/HCl buffer, pH 8.2, containing 150 mM-NaCl and 30 mM-Ca^{2+}.

Haemagglutination test

Haemagglutination tests (Sarkar *et al.*, 1984) were performed by the serial dilution technique in a Takatsy microtitrator (Cooke Engineering Co., Alexandria, VA, U.S.A.) with 2% (v/v) rabbit erythrocytes suspension in saline (0.9% NaCl). Titres for native as well as modified lectin were scored after 1 h incubation at 25 °C and 10 °C separately. Minimal titre concentration required for the modified protein was compared with that for the native protein and the relative haemagglutinating (HA) activity was calculated.

Binding to SSM-Sepharose 4B

Native or modified protein was loaded on to an SSM-Sepharose 4B column (1 cm × 4 cm; 115 nmol of sialic acid/ml of gel) equilibrated with buffer A and their binding to the affinity matrix was measured as described by Basu *et al.*, 1986.

Carbohydrate analyses

A gas-chromatographic method was used for the analysis of neutral sugars, and an amino acid analyser was used for the amino sugars.

The trimethylsilyl (Me_3Si)-derivative method was performed as described by Chambers & Clamp (1971). The agglutinin (0.5 mg) was methanolysed in 500 μl of 1 M-HCl in methanol under N_2 at 85 °C for 4 h, with mannitol as an internal standard. The 'methanolysate' was then dried under a stream of N_2 and the methyl glycosides were converted into their Me_3Si derivatives by the addition of the silylating reagent (trimethylchlorosilane/hexamethyldisilazane/pyridine, 1:3:9, by vol.) and incubation for 10 min at 25 °C. The Me_3Si derivatives were separated with N_2 by a capillary g.l.c. system on a WCOT fused-silica column (25 m × 0.32 mm internal diam.) with a coating of CP-SIL8 (Chrompack, Middeburg, The Netherlands). The gas chromatograph was a Varian 3300 instrument fitted with flame-ionization detection, and the peaks were integrated by an attached Varian 4290 integrator.

Amino sugars were determined on the amino acid analyser after hydrolysis of the glycoprotein at 100 °C for 24 h in 3 M-toluene-4-sulphonic acid under N_2. This procedure gives recoveries in excess of 90% (Allen & Neuberger, 1975).

Amino acid analysis

Amino acids were analysed on a Locarte amino acid analyser by reaction with ninhydrin after hydrolysis in 3 M-toluene-4-sulphonic acid under N_2 for 24 h at 110 °C with *p*-fluorophenylalanine as an internal standard. Half-cystine values were obtained from samples which had been previously oxidized with performic acid (Hirs, 1967). For further details of the analytical systems which were used, see Allen *et al.* (1976). Tryptophan was determined spectrophotometrically (Edelhoch, 1967; Bredderman, 1974).

Removal of the modifying reagents

The modified protein samples were freed of excess reagents by exhaustive dialysis against 50 mM-Tris/HCl, pH 8.2, containing 150 mM-NaCl. Each and every step of reaction was monitored by appropriate control experiments.

Chemical modification of ATN_H

All the reactions were performed under mild conditions and with specific reagents that could modify only particular amino acids.

***(a)* Reductive alkylation of protein ATN_H.** Reductive methylation of ATN_H was done essentially as described by Means & Feeney (1968). To the lectin solution (0.2 mg/ml) in 200 mM-sodium borate buffer, pH 9.2, at 0 °C was added 1 μmol of aq. $NaBH_4$, followed by rapid addition of 2 μmol of aq. formaldehyde. The addition of $NaBH_4$ and formaldehyde was repeated twice at 15 min intervals. The reaction mixture was incubated for 1 h, acidified and dialysed extensively. The extent of modification of the lysine ϵ-amino groups was assessed by using 2,4,6-trinitrobenzenesulphonic acid (Habeeb, 1967).

***(b)* Citraconylation of ATN_H.** Citraconylation of ATN_H was carried out by the method as described by Dixon & Perham (1968). To the lectin solution (0.2 mg/ml in 0.05 M-Tris/HCl buffer, pH 8.0) was added a 300-fold molar excess of the citraconic anhydride with constant stirring. The solution was stirred at 4 °C for 1 h, and the pH was maintained at 8.0 by the addition of

0.1 M-NaOH. The decitraconylation was carried out by leaving the citraconylated protein overnight at 4 °C at pH 3.0. The percentage modification in this reaction was monitored by determination of unmodified amino groups with 2,4,6-trinitrobenzenesulphonic acid as described by Habeeb (1967).

(c) Oxidation of ATN_H with NBS. Oxidation of ATN_H with NBS was carried out by the method of Spande *et al.* (1966), both in the absence of urea and after denaturing the protein with 10 M-urea. Aq. NBS (1 mM) was added in 10 μl portions to a solution of protein (0.2 mg/ml and 0.15 mg/ml respectively) in 100 mM-acetate buffer, pH 4, at 25 °C. The number of tryptophan groups modified were calculated by the method of Spande & Witkop (1967).

(d) Treatment with 2-hydroxy-5-nitrobenzyl bromide. Modification with 2-hydroxy-5-nitrobenzyl bromide was done as described by Horton & Koshland (1972) in the presence of 8 M-urea. In a typical experiment, to a 0.2 mg/ml protein solution in 0.15 M-acetic acid, pH 2.7, was added 0.5 mg of 2-hydroxy-5-nitrobenzyl bromide in 0.1 mg of dry acetone. The pH was maintained below 3 by the addition of acetic acid. The modified protein was removed from the excess of reagent on a Sephadex G-25 column (6 cm × 0.5 cm) equilibrated with 0.15 M-acetic acid.

(e) Modification of tyrosine residues in ATN_H with *N*-acetylimidazole. For the chemical modification as well as the determination of the number of exposed and buried tyrosine residues in ATN_H, the procedure of Riordan *et al.* (1965) was used, both for the native protein and after denaturation with 10 M-urea. In this experiment, the lectin solution (0.2 mg/ml) was treated with a 60-fold molar excess of *N*-acetylimidazole in 10 mM-Tris/HCl buffer, pH 7.5, at 25 °C for 1 h. After the reaction, the modified protein was dialysed to remove excess reagents. The protein was de-*O*-acetylated with hydroxylamine.

(f) Modification of histidine residues with diethyl pyrocarbonate. Modification of histidine residues in ATN_H was carried out by the method of Melchior & Fahrney (1970). In a typical experiment, diethyl pyrocarbonate was added in 3-fold molar excess over the histidine content to a 0.2 mg/ml protein solution in 20 mM-sodium phosphate buffer, pH 7.2, at 25 °C. The extent of modification of histidine residues was determined as described by Anderson & Ebner (1979).

(g) Modification of arginine residues with cyclohexane-1,2-dione. The arginine residues of ATN_H were modified essentially as described by Patthy & Smith (1975). To the lectin solution (0.2 mg/ml) in 10 mM-triethanolamine buffer, pH 8.0, was added cyclohexane-1,2-dione in a 1:1 ratio by weight and the reaction was allowed to proceed for 12 h in dark. Excess reagent was subsequently removed by dialysis.

(h) Treatment with phenyl glyoxal. Modification with phenyl glyoxal was carried out (Mukherjee & Bhaduri, 1986) in 0.02 M-sodium phosphate buffer, pH 8.0, at 28 °C. The phenyl glyoxal was dissolved in dimethyl sulphoxide. Control experiments without modifying reagents were always run. The reaction mixture was incubated for 30 min to ensure complete modification. The modified protein was passed through a Sephadex G-50 column to remove excess reagents.

(i) Modification of carboxy groups in ATN_H. The carboxy groups of the lectin were modified as described by Hoare & Koshland (1967). To the lectin solution (0.2 mg/ml) in 10 mM-Tris/HCl buffer, pH 7.0, containing 8 M-urea, 5 mg of glycine methyl ester was added. To this reaction mixture 1 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide was added. The solution was adjusted in the pH range 4.6–4.9 with 0.1 M-NaOH and 0.1 M-HCl, incubated for 1 h at room temperature, dialysed 16 h against water, treated with 0.5 M-hydroxylamine at pH 7.0 for 7 h (Carraway & Koshland, 1968), during which time the protein absorption at 278 nm increased 10–20%, and then dialysed extensively.

(j) Alkaline β-elimination reaction on ATN_H. ATN_H (0.2 mg/ml) was treated with aq. KOH, dimethyl sulphoxide and ethanol to give a final solution of 170 mM-KOH having a dimethyl sulphoxide/water/ethanol ratio of 5:4:1 (by vol.). This solution was incubated in a

Table 1. Amino acid and carbohydrate composition of purified ATN_H

Results are based on an M_r of 240000, including 21% (w/w) carbohydrate. For analytical methods, see the text.

Amino acid	Composition	
	Residues/100 residues	mol/mol of glycoprotein (M_r 240000)
Asx	10.7	194
Thr	5.1	89
Ser	7.8	137
Glx	10.4	184
Pro	8.4	147
Gly	8.7	153
Ala	8.5	150
½-Cys*	1.3	23
Val	6.9	121
Met	0.9	15
Ile	3.3	58
Leu	6.8	120
Tyr	2.7	47
Phe	3.7	65
Trp†	4.3	75
His	3.2	56
Lys	4.0	70
Arg	3.3	56
GlcNAc	2.6	45
GalNAc	2.0	35
Fuc	2.2	39
Xyl	5.6	96
Man	3.3	58
Gal	2.0	35
Total carbohydrate	17.7	308

* Determined separately as cysteic acid after hydrolysis of performic acid-oxidized sample (Hirs, 1967).

† Determined by a spectrophotometric method (Edelhoch, 1967; Bredderman, 1974).

Table 2. Effect of chemical modification on HA activity and carbohydrate-binding properties

The various reagents used to modify groups are tabulated. The reaction procedures were those described in the Materials and methods section. Results for immunochemical properties and HA activities are with respect to the native protein. '+' denotes the binding, and '−' a lack of binding, of ATN_H to the affinity matrix; n.d. not determined.

Modification	Residues modified	No. of residues modified/ molecule of ATN_H	Immunochemical properties	Relative HA activity		Binding to SSM–Sepharose-4B matrix
				25 °C	10 °C	
None (native protein)	–	–	Unchanged	1	1	+
Reductive alkylation	Lysine	51	Unchanged	0.43	0.50	+
Citraconic anhydride	Lysine	63	Unchanged	0.45	0.55	+
Decitraconylation	–	–	–	1	1	+
NBS-mediated Oxidation	Tryptophan					
In the absence of urea		35	Changed	0	0	–
In the presence of urea		49	Changed	0	0	–
2-Hydroxy-5-nitrobenzyl bromide	Tryptophan	63	Changed	0	0	–
N,O-Diacetylation	Tyrosine					
In the absence of urea		12	Unchanged	1	0.9	+
In the presence of urea		47	Unchanged	0.7	0.7	+
Diethyl pyrocarbonate	Histidine	28	Changed	0	0	–
Cyclohexanedione	Arginine	n.d.	Unchanged	0.7	1	+
Phenyl glyoxal	Arginine	n.d.	Unchanged	0.8	1	+
Glycine methyl ester	Carboxy	n.d.	Unchanged	0.17	0.1	Slight

water bath at 45 °C for 1 h, neutralized with HCl (Downes *et al.*, 1973), then dialysed. The percentage of carbohydrate in the modified ATN_H was estimated by the phenol/sulphuric acid method of Dubois *et al.* (1956).

(*k*) Oligosaccharide liberated by alkaline borohydride treatment. ATN_H (0.2 mg/ml) was treated with 50 mM-NaOH/2 M-$NaBH_4$ for 18 h at 45 °C by the method of Hatcher *et al.* (1977). The excess borohydride was eliminated by addition of acetic acid at 4 °C. The sample was dialysed and the percentage of carbohydrate in the modified ATN_H was estimated by the method of Dubois *et al.* (1956).

Determination of sialidase activity of ATN_H

$Achatinin_H$ (55 μg/ml) was treated with fetuin by the method of Stockert *et al.* (1974). The sialic acid content of fetuin was determined by the thiobarbituric acid assay of Warren (1959) as modified by Saifer & Gerstenfeld (1962). The control experiment was done with neuraminidase instead of ATN_H.

Fluorescence emission measurements

Fluorescence measurements were carried out on a Perkin–Elmer MPF-44 B fluorescence spectrophotometer at 25 °C. The samples were excited at 285 nm and the emission spectra were recorded from 300–400 nm.

C.d. analysis

The c.d. spectra of purified ATN_H (0.20 mg/ml) and its modified derivatives (0.20 mg/ml) were measured at 25 °C in cells of 2 mm and 1 mm path length of 2 ml capacity with a Jasco J-20 A recording spectropolarimeter. These data are expressed in terms of mean residue ellipticities ($[\theta]_{m.r.w.}$) expressed in degrees·cm^2·$dmol^{-1}$. All recordings were made in 0.05 M-Tris/HCl buffer/0.9% NaCl containing 0.05 M Ca^{2+}.

RESULTS AND DISCUSSION

The present report describes a modified method for the purification of a highly specific sialic acid-binding agglutinin from the haemolymph of *Achatina fulica*. Although the yield of ATN_H is 20% lower than that obtained in our earlier-reported method, the present procedure is much simpler. The specific activity of the purified lectin increased approx. 800-fold as compared with that of the crude extract. In this new purification procedure the protein was eluted simply by elevating the temperature to 30 °C and withdrawing Ca^{2+} from the eluting buffer, so it was possible to avoid several dialysis steps, since the buffer used for the specific-elution purpose is devoid of citrate. This therefore provides a very simple and inexpensive method for the isolation of the protein. This could also be a very useful method to purify other metal-binding proteins. The affinity-purified ATN_H was found to be homogeneous by several techniques. ATN_H can agglutinate rabbit red blood cells at both 10 °C and 25 °C, but the agglutination titre is somewhat higher at 10 °C in the presence of Ca^{2+}. The molecule is composed of 16 non-covalently bound identical subunits (M_r 15000) which correspond to a glycoprotein of native M_r 240000 (Basu *et al.*, 1986). The amino acid and carbohydrate composition of ATN_H is given in Table 1. Aspartic acid and glutamic acid, with their amides, are the most abundant amino acids, together accounting for 22% of the residues. The pI of ATN_H (6.2; Basu *et al.*, 1986) is in agreement with this idea, if one assumes that the values for glutamate and aspartate are due to both for the free acids and their amides in the intact protein. An unusually high content of acidic amino acid residues seems to be a general characteristic of sialic acid-binding lectins. Half-cystine or cystine accounts for 1.3% of the residues, which would permit some degree of disulphide-bonding. The 11 disulphide bonds corresponding to 23 mol of half-cystine are not involved in subunit

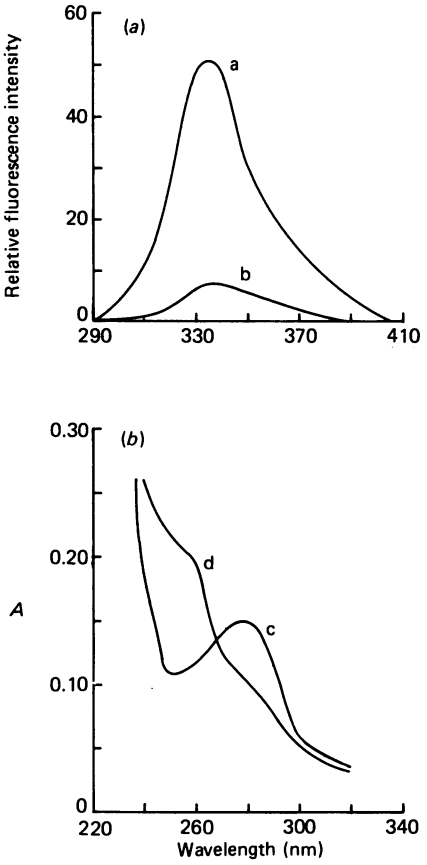

Fig. 1. Effect of modification of tryptophan residues on the fluorescence and on the u.v. absorption of ATN_H

(*a*) Decrease in the relative fluorescence intensity of tryptophan emission with the modification of tryptophan residues is shown. The emission maximum is observed at 335 nm for excitation at 285 nm. Curve a, native protein; curve b, 49 tryptophan residues modified/molecule of ATN_H. (*b*) The change in absorbance with the modification of tryptophan residues observed in the native protein is plotted. Curve c, native protein; curve d, 49 tryptophan residues modified/molecule of ATN_H.

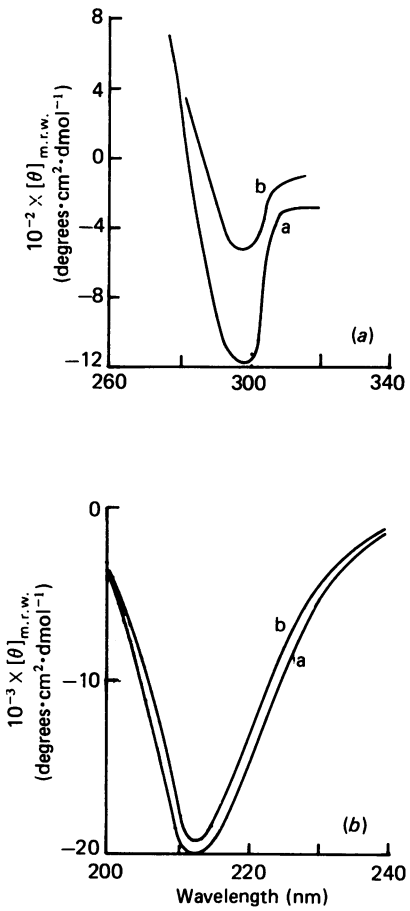

Fig. 2. Effect of the modification of tryptophan residues on the c.d. of ATN_H at 25 °C

(*a*) Near-u.v. region (260–340 nm): curve a, native protein; curve b, modified protein. (*b*) C.d. spectra of native (curve a) and modified (curve b) agglutinin were plotted in the far-u.v. region (200–240 nm).

interactions. The ATN_H is a glycoprotein having 21% (w/w) carbohydrate. Xylose is the most abundant sugar (6%), which is quite noteworthy, since evidence for xylose as a constituent of *N*-glycosidic carbohydrate chains has been reported (Van Kuik *et al.*, 1985) in the α-haemocyanin of *Helix pomatia* (Roman snail) and in a trace amount (0.6%) in the cold agglutinin from *Achatina fulica* (Mitra *et al.*, 1987). It may be that xylose will be found to be a common constituent of molluscan glycoproteins. To the best of our knowledge this is the first report of a snail lectin that contains such a high content of xylose and fucose (2.7%). Besides that it contains *N*-acetylglucosamine (4.1%), *N*-acetylgalactosamine (3.2%), fucose (2.7%) and mannose (4.35%). The low galactose content (2.6%) of ATN_H, in contrast with the high galactose content (14.4%) of another lectin from this snail (Mitra *et al.*, 1987), is also noteworthy.

The effect of chemical modification on the HA activity, carbohydrate-binding properties and the extent of modification of the various amino acid residues in ATN_H are summarized in Table 2. These studies revealed the essential involvement of tryptophan, histidine (28 residues), carboxy and lysine residues in the binding activity of ATN_H. Reductive methylation resulted in 73% modification, and citraconylation resulted in 90% modification of the amino groups in lysine residues. This modification of ε-amino groups resulted in an almost 50% decrease in the HA activity as well as in binding to the affinity matrix. Decitraconylation led to the release of the modified amino groups and caused biological activity to be regained. The lectin lost almost 80–90% of its HA activity as well as of its matrix-binding property when carboxy groups were esterified. This confirms that aspartic acid and glutamic acid residues also play an important role in the biological activity of ATN_H.

Modification of tryptophan and histidine (28) residues led to a drastic loss of activity. The oxidation of ATN_H with NBS is instantaneous and quantitative at acidic pH, with a large decrease in A_{280} accompanying the transformation of the indole to oxindole chromophore. NBS is known to attack both tyrosine and tryptophan residues in the pH range 3.4–4.0. In the present study, under the experimental conditions described, the reagent modified only the tryptophan residues. In all, 35 residues

Table 3. Carbohydrate modification in ATN_H

The reaction procedures used were those described in the Materials and methods section. The extent of carbohydrate modification is expressed as a percentage retention of carbohydrate residues in the modified ATN_H with respect to the native ATN_H. Carbohydrate was estimated by the method of Dubois *et al.* (1956). '+' and '−' denote positive and negative HA activity respectively

Modification	Relative agglutination: Room temperature (25 °C)	Relative agglutination: Cold (10 °C)	Carbohydrate (%)
None	+	+	100
Alkaline borohydride	−	−	60
Alkaline β-elimination	−	−	50

were modified in absence of urea, and when the reaction was carried out in presence of urea, 49 residues were modified. However 63 tryptophan residues were modified when 2-hydroxy-5-nitrobenzyl bromide was used. This is borne out by the absorption measurements (Fig. 1*b*), which clearly reveal that tyrosine residues were not modified as the isosbestic point at 268 nm remains unchanged (Spande & Witkop, 1967; Lowe & Whitworth, 1974), even after the modification of tryptophan residues in the presence of urea. Owing to these modifications the carbohydrate-binding ability and the HA activity of ATN_H were completely abolished. There was a marked decrease in the fluorescence emission as the tryptophan residues of ATN_H were modified, both in the absence and in the presence of urea. On excitation at 285 nm, 82% and 85% losses in fluorescence emission were found at 335 nm when 35 and 49 tryptophan residues/mol of ATN_H were modified respectively, compared with the native ATN_H (Fig. 1*a*). The fluorescence emission maxima at 335 nm is typical of tryptophan emission in proteins, indicating that the fluorescence is solely due to tryptophan residues exposed on the protein's surface (Kelly & Von Hippel, 1976; Herrmann & Behnke, 1980).

The c.d. spectra of the native protein showed a negative band, centred at 214 nm, of ellipticity value -20000 degree·cm^2·$dmol^{-1}$ as demonstrated by the curve in Fig. 2(*b*). The near-u.v. region was characterized by a broad band at 290–300 nm and a peak, centred at 295 nm, of ellipticity value -1168 degrees·cm^2·$dmol^{-1}$. Nevertheless, there was a decrease in molar ellipticity in the chromophoric region as the tryptophan residues were oxidized (Fig. 2*a*). This decrease is presumably due to modified tryptophan residues (Jirgensons, 1973). The loss in biological activity could be due to a change in either the secondary structure or the state of aggregation of the protein. The former possibility is ruled out, since the c.d. spectrum of the modified agglutinin in the far-u.v. region was almost similar to that of the native agglutinin (Fig. 2*b*). Thus the loss of activity was not due to a drastic conformational change. The evidence for the presence of tryptophan, glutamic acid and aspartic acid residues in the saccharide binding site of various lectins has been reported (Goldstein & Hayers, 1978; Jordan

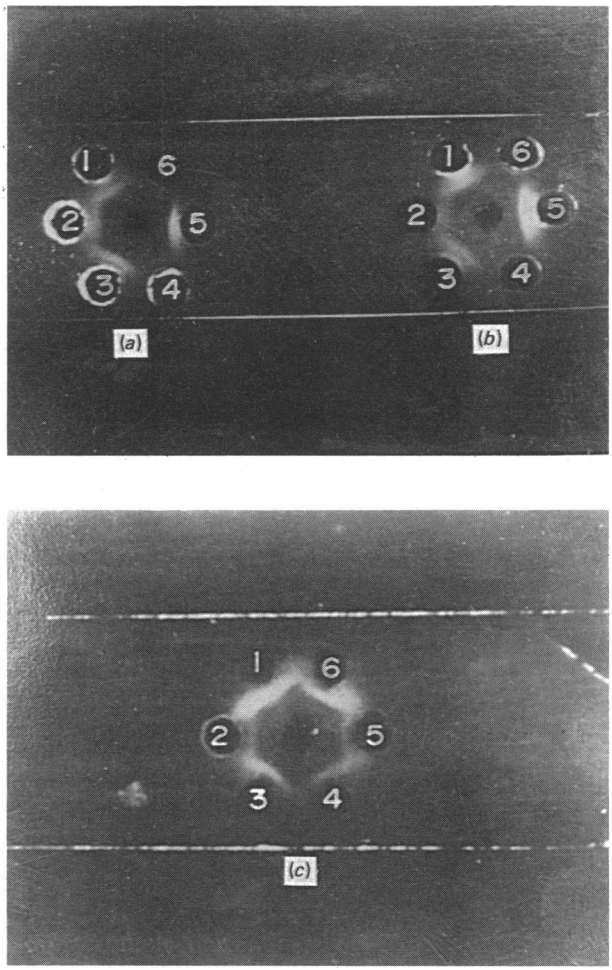

Fig. 3. Immunodiffusion test for various modifications of ATN_H

In each case the centre well contains anti-haemolymph serum. (*a*) Wells 1, 3 and 5 are appropriate controls of native ATN_H for wells 2, 4 and 6, where well 2 is lysine-modified ATN_H, well 4 is tryptophan residues modified in absence of urea and well 6 is tryptophan residues modified in presence of urea. (*b*) Wells 1, 3 and 5 are appropriate controls of native ATN_H for wells 2, 4 and 6, where well 2 is carboxy-modified ATN_H and wells 4 and 6 are histidine-modified. (*c*) Wells 1, 3 and 5 are appropriate controls of native ATN_H for wells 2, 4 and 6, where well 2 is tyrosine-modified ATN_H in the presence of urea, well 4 is tyrosine-modified in absence of urea, and well 6 is ATN_H with arginine residues modified.

et al., 1977). In potato (*Solanum tuberosum*) lectin, both tyrosine and tryptophan residues were implicated in carbohydrate-binding activity (Ashford *et al.*, 1981).

Acetylation of the lectin with *N*-acetylimidazole resulted in a preferential modification of tyrosine residues (Riordan *et al.*, 1965). Such treatment of ATN_H resulted in the modification of 12 groups in the absence of urea and 47 in the presence of urea (100% modification). Despite these modifications, no change in the HA activity or in the matrix-binding ability was observed. Similarly ATN_H was treated with cyclohexane-1,2-dione and with another arginine-specific reagent (phenyl glyoxal) and in both cases modification did not produce any change in the specific activity of the lectin. The matrix-binding

property of arginine-modified ATN_H remained unaltered. These observations rule out the possibility of these residues being involved in biological activity.

The carbohydrate groups of the purified ATN_H were removed by a β-elimination reaction. Carbohydrate analysis of modified ATN_H by the phenol/sulphuric acid method showed that 50% of the total carbohydrate residues were removed by this reaction after treatment for 1 h at 45 °C. As the β-elimination reaction removes carbohydrate moieties, it can be assumed that the sugars are linked to protein of ATN_H *O*-glycosidically and that these moieties play an important role in binding. On alkaline borohydride treatment, 60% of the carbohydrate components were removed. From Table 3 it is clear that the HA activity of modified ATN_H at both 25 °C and 10 °C was lost completely. The carbohydrate-modified agglutinin also failed to bind to the affinity matrix. The loss of reactivity could be due to the loss of carbohydrate or to denaturation of the polypeptide by alkaline digestion.

The perfect fusion of precipitin lines in immunodiffusion suggests a complete cross-reactivity between the native and the modified protein samples, suggesting that the ability of the protein to bind the antibody did not change appreciably; hence the modification did not cause any drastic conformational change in the secondary structure of the modified protein (Fig. 3), except in the case of tryptophan- and histidine-modified agglutinin.

ATN_H lacks sialidase activity as determined by the procedure described in the Materials and methods section.

The present study strongly suggests that tryptophan and histidine residues are probably essential for saccharide binding, matrix binding and the immunological activity of ATN_H. However, the role of carboxy and lysine residues cannot be ruled out. Besides that, the carbohydrate part of the molecule also plays an important role. To the best of our knowledge this is the first report of such a lectin; it will be useful as an analytical tool in studying the biological functions of several sialoglycoconjugates of cellular membranes and in their affinity-purification. Moreover, it could be used as a powerful reagent in studies of changes in the red-blood-cell surface with the degree of *O*-acetylation in transformation and other alterations in the environment of cells.

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