Chemical modification studies on a lectin from *Saccharomyces cerevisiae* (baker's yeast)

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The effect of chemical modification on a galactose-specific lectin isolated from a fatty acid auxotroph of *Saccharomyces cerevisiae* was investigated in order to identify the type of amino acids involved in its agglutinating activity. Modification of 50 free amino groups with succinic anhydride or citraconic anhydride led to an almost complete loss of activity. This could not be protected by the inhibitory sugar methyl α -D-galactopyranoside. Treatment with *N*-bromosuccinimide and *N*-acetylimidazole, for the modification of tryptophan and tyrosine residues, did not affect lectin activity. Modification of carboxy groups with glycine ethyl ester greatly affected lectin activity, although sugars afford partial protection. Modification of four thiol groups with *N*-ethylmaleimide was accompanied by a loss of 85% of the agglutinating activity, and two thiol groups were found to be present at the sugar-binding site of the lectin. Modification of 18 arginine residues with cyclohexane-1,2-dione and 26 histidine residues with ethoxyformic anhydride led to a loss of lectin activity. However, in these cases, modification was not protected by the abovementioned inhibitory sugar, suggesting the absence of these groups at the sugar-binding site. In all the cases, immunodiffusion studies with modified lectin showed no gross structural changes which could disrupt antigenic sites of the lectin.

INTRODUCTION

Lectins are multivalent carbohydrate-binding haemagglutinating proteins of non-immune origin (Goldstein *et al.*, 1980). Binding of lectins to cell-surface carbohydrates induces such interesting effects as blood-group-specific haemagglutination, mitogenic stimulation of lymphocytes, preferential agglutination of tumour cells etc. (Novogrodosky & Katchalski, 1971; Cuatrecasas & Tell, 1973; Lis & Sharon, 1973; Goldstein & Hayes, 1978).

The haemagglutinating activity of lectins is attributed to their interaction with specific sugars. Specific amino acid residues are essential for maintaining the carbohydrate-binding and haemagglutinating activities of lectins. Identification of these amino acid residues is a prerequisite in investigations on the structure–function relationships of lectins. Chemical modification with group-specific modifying agents in the presence or absence of interacting ligand (in the present case an inhibitory sugar) provides a general approach for identification of the amino acid residues present in the functional or active site of any protein, including lectins.

Recently a galactose-specific lectin has been reported from this laboratory from a fatty acid auxotroph of baker's yeast (*Saccharomyces cerevisiae*). It is a glycoprotein of M_r approx. 320 000 containing 60% carbohydrate and having a pI of 4.2 (Basu *et al.*, 1986). The present paper describes the further characterization of this lectin, including amino acid and sugar analysis, and the effect of different chemical modifying agents on the biological, as well the immunochemical, properties of the protein. This has been done with a view to gaining information on the structure–function relationship within the novel lectin, enabling us to make comparisons with other lectins of diverse origin.

EXPERIMENTAL

Materials

2,4,6-Trinitrobenzenesulphonic acid, *N*-acetylimidazole, *N*-bromosuccinimide, succinic anhydride, citraconic anhydride, glycine ethyl ester, cyclohexane-1,2-dione, ethoxyformic anhydride, *N*-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoic acid), dansyl chloride, Brij 58 and methyl α -D-galactopyranoside were obtained from Sigma; Sephadex G-25 and G-200 were obtained from Pharmacia Fine Chemicals; oleic acid was obtained from Nu Chek Prep; Trisil-Z was obtained from Pierce; 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide was obtained from Aldrich; and *N*-ethyl[2,3- 14 C]maleimide was obtained from Amersham International. All other reagents used were of analytical grade.

Preparation of yeast lectin

The fatty acid auxotroph KD 115 of *Saccharomyces cerevisiae* was generously given by the Yeast Genetic Stock Center, Berkeley, CA, U.S.A. It was grown in a liquid medium supplemented with oleic acid and Brij 58 for 48 h, and the lectin was purified from the culture broth as described by Basu *et al.* (1986). It was further clarified by centrifugation at 160 000 g for 90 min.

Haemagglutinating activity

Haemagglutination tests were carried out by the serial-dilution technique (Kabat & Mayer, 1961) with 0.15 M-NaCl/20 mM-sodium phosphate, pH 7.2, in microtitre plates with a 3% suspension of rabbit erythrocytes. The end point was visually estimated after 90 min, and the lowest dilution showing haemagglutinating activity was used to calculate specific activity of the lectin. The relative agglutinating activity of the chemi-

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cally modified lectin samples was expressed as a percentage of the specific activity of the native lectin.

Amino acid analysis

Amino acid analyses of native and modified lectin were performed as described by Spackman *et al.* (1958) in a Beckman Multichrom liquid-column chromatograph. Lectin samples were hydrolysed with 6 M-HCl at 110 °C for 24, 48 and 72 h in sealed evacuated tubes. For each hydrolysate, duplicate analyses were performed. Tryptophan was estimated spectrophotometrically by the method of Edelhoch (1967). Free thiol groups were determined by the method of Ellman (1959).

Sugar analysis

Neutral and amino sugars were analysed by g.l.c. of their trimethylsilyl derivatives as described by Clamp *et al.* (1972). Briefly, methanolysis of the samples was carried out with methanolic 1.5 M-HCl, followed by *N*-acetylation with acetic anhydride and silylation with Tri-Sil Z. Samples were then injected into a Shimadzu gas chromatograph (GCR1A) fitted with a data processor (RPRG1) using a 3% SE 30 column (1.5 m × 3 mm), N₂ at a flow rate of 50 ml/min, an initial temperature of 140 °C and a programming rate of 0.5 °C/min. Peaks were identified by using standard sugars subjected to g.l.c. after the treatment described above.

Determination of the *N*-terminal amino acid

The *N*-terminal amino acid of the lectin was determined by the dansyl chloride method (Hartley & Massey, 1956). The dansyl amino acid was identified by t.l.c. on silica-gel G by using standard dansyl amino acids and the following solvent systems: (i) chloroform/ethanol/acetic acid (38:4:3, by vol.); (ii) butan-1-ol/pyridine/acetic acid/water (15:10:3:12, by vol.); (iii) chloroform/benzyl alcohol/acetic acid (70:30:3, by vol.); and (iv) chloroform/2-methylpropan-2-ol/acetic acid (6:3:1, by vol.).

Immunodiffusion

Ouchterlony plates of 2% agar in 0.15 M-NaCl containing 0.02% azide were prepared. Lactose was used to inhibit the interaction of galactose residues of serum glycoproteins or agar with the lectin.

Gel filtration on Sephadex G-200

Gel filtration was carried out on a Sephadex G-200 column (1.6 cm × 70 cm) at a flow rate of 1.5 ml/min. *M_r* markers used were catalase, aldolase, bovine serum albumin and ovalbumin.

Modification of amino groups

Modifications of amino groups with succinic anhydride and citraconic anhydride were carried out by the methods of Habeeb *et al.* (1958) and Dixon & Perham (1968) respectively. The lectin sample (3 mg) was treated with 500-fold molar excess of the modifying reagent in saturated NaHCO₃ solution. Unblocking of the amino groups of the citraconylated lectin was carried out by dialysis against 0.1 M-acetic acid overnight at 4 °C. The extent of amino group modification was estimated by determining the amino groups after modification with 2,4,6-trinitrobenzenesulphonic acid as described by Habeeb (1967).

Modification of carboxy groups

The lectin sample (5 mg) was dissolved in 1 ml of 1 M-glycine ethyl ester (also containing 8 M-urea where mentioned) and 100 µl of 1.1 M-carbodi-imide was added, the pH being maintained at 4.75. The mixture was stirred at room temperature (Hassing *et al.*, 1971), and excess reagent was removed by gel filtration on a Sephadex G-25 column equilibrated with 0.15 M-NaCl/20 mM-sodium phosphate, pH 7.2, followed by dialysis against the same buffer.

Modification of histidine residues

Modification of histidine residues with ethoxyformic anhydride was carried out as described by Andersen & Ebner (1979). The lectin sample (1 mg/ml) in 20 mM-sodium phosphate buffer, pH 7.2, was treated with ethoxyformic anhydride at a final concentration of 3-fold molar excess over the histidine content at 25 °C. At appropriate time intervals, aliquots were removed and the reaction was terminated by the addition of an equal volume of 10 mM-histidine. Samples were dialysed against 20 mM-phosphate buffer, pH 7.2, in order to get rid of excess reagent as well as ethoxyformylated histidine. Modification of the histidine residues was reversed by treatment with 0.05 M-hydroxylamine for 10 min, followed by dialysis to remove the products. The extent of histidine modification was determined spectrophotometrically at 242 nm.

Modification of arginine residues

Modification with cyclohexane-1,2-dione was performed by the method of Patthy & Smith (1975). The lectin sample (5 mg/ml) in 0.2 M-sodium borate buffer, pH 9.0, was treated with 0.05 M reagent under an N₂ atmosphere at 37 °C for 4 h in the dark. The reaction was terminated by adding 1 ml of 5% (v/v) acetic acid. For amino acid analysis, modified lectin was dialysed exhaustively against 5% (v/v) and then 1% (v/v) acetic acid. This acid treatment was found to stabilize the interaction in the absence of borate. Reversal of this modification was achieved by the treatment of the modified lectin with 1 M-hydroxylamine at 37 °C for 4 h under N₂, followed by dialysis to remove excess reagent.

Modification of thiol groups

Modification was performed by the method of Riordan & Vallee (1972). Lectin solution in 20 mM-sodium phosphate buffer, pH 7.2, was treated with 1 mM-*N*-ethyl[2,3-¹⁴C]maleimide (sp. radioactivity 25 µCi/mmol) at 25 °C for 30 min. The reaction was terminated by passing the reaction mixture through a Sephadex G-25 column, followed by dialysis. To quantify the extent of modification, 100 µl of the modified lectin solution was placed on a Whatman filter paper, washed with ice-cold 10% (w/v) trichloroacetic acid to precipitate protein and then with ethanol, and counted for radioactivity in an LKB liquid-scintillation counter (Minibeta 1211), a toluene-based scintillant containing 3 g of PPO (2,5-diphenyloxazole) and 100 mg of POPOP [1,4-bis(5-phenyloxazol-2-yl)benzene] being used. Thiol-group modification was also quantified spectrophotometrically by reaction with 1 mM-5,5'-dithio-bis-(2-nitrobenzoic acid) as described by Ellman (1959).

Modification of tryptophan residues

Modification of tryptophan residues was carried out with *N*-bromosuccinimide and monitored spectrophotometrically (Spande & Witkop, 1967). Lectin solution in sodium acetate buffer, pH 4.0, was treated with *N*-bromosuccinimide at 25 °C, and excess reagent was removed by dialysis.

Modification of tyrosine residues

Modification of tyrosine residues was carried out with *N*-acetylimidazole and monitored spectrophotometrically (Riordan *et al.*, 1965). The amino groups of the lectin were citraconylated by citraconic anhydride, and it was then treated with 300-fold molar excess of *N*-acetylimidazole in 0.01 M-sodium phosphate buffer, pH 7.4, at 15 °C for 1 h. The reaction was terminated by passing the mixture through Sephadex G-25 and then decitraconylated before testing its activity.

Protection by methyl α -D-galactopyranoside

Chemical modification with group-specific modifying agents was carried out both in the presence and in the absence of 0.2 M-methyl α -D-galactopyranoside. The samples were desalted by gel filtration on Sephadex G-25 and dialysed before determining the loss of agglutinating activity.

RESULTS AND DISCUSSION

Amino acid analysis

The amino acid composition of the yeast lectin is shown in Table 1. In common with most lectins, the yeast lectin is rich in acidic and hydroxy amino acids (Lis & Sharon, 1972). Four cysteine residues were found to be present [as determined by the method of Ellman (1959)]. However, the number of cystine residues was not estimated.

Sugar analysis

Analysis of sugars by g.l.c. showed that, of the carbohydrate present, the lectin contains 49% mannose and 50% hexosamine (amino sugars include those present in the *N*-acetylated form also).

N-Terminal amino acid analysis

Arginine was found to be the only *N*-terminal amino acid (as determined by the dansyl chloride method).

Modification of free amino groups

Succinylation or citraconylation modified 53% of the free amino groups of the yeast lectin, and this was accompanied by a complete loss of its agglutinating

Table 1. Amino acid composition of purified yeast lectin

Amino acid	Composition (residues/molecule)	
	Calculated	Nearest integer
Aspartic acid	97.1	97
Threonine	85.4	85
Serine	84.9	85
Glutamic acid	83.2	83
Proline	50.3	50
Glycine	86.9	87
Alanine	97.9	98
Valine	66.8	67
Half-cystine	4	4
Methionine	9.5	10
Isoleucine	81.4	81
Leucine	57.2	57
Tyrosine	16.4	16
Phenylalanine	29.5	30
Tryptophan	15.8	16
Histidine	30.3	30
Lysine	93.6	94
Arginine	30.3	30

Table 2. Effect of modifying agents on the yeast lectin

The immunochemical properties and M_r were unchanged throughout. Abbreviation used: n.d., not determined.

Chemical treatment	Type of residues modified	No. of residues modified	Agglutinating activity (%)
Citraconic anhydride	Amino	53.7	0
Citraconic anhydride, then acetic acid	Amino	0	90
Succinic anhydride	Amino	50	0
Ethoxyformic anhydride	Histidine	26	0
Ethoxyformic anhydride, then 1 M-hydroxylamine	Histidine	4	90
Cyclohexane-1,2-dione	Arginine	18	25
Cyclohexane-1,2-dione, then 1 M-hydroxylamine	Arginine	6	85
Glycine ethyl ester and carbodi-imide	Carboxy	N.D.	16
Glycine ethyl ester, carbodi-imide and 8 M-urea	Carboxy	N.D.	50
<i>N</i> -Ethylmaleimide	Thiol	4	5
5,5'-Dithiobis-(2-nitrobenzoic acid)	Thiol	4	2
<i>N</i> -Acetylimidazole	Tyrosine	N.D.	100
<i>N</i> -Bromosuccinimide	Tryptophan	N.D.	100

activity (Table 2). Decitraconylation of the modified lectin led to a 90% recovery of its activity. The time-dependent modification with succinic anhydride showed that modification of the first 24 amino groups led to a loss of 50% lectin activity (Fig. 1), and at least 24 amino groups were found to be involved in the agglutinating activity (Fig. 2). Modification of the amino groups may lead to change in conformation and/or dissociation of subunits (Habeeb *et al.*, 1958; Gunther *et al.*, 1973). However, in this case the M_r of the modified lectin was the same as that of the native lectin when determined by gel filtration on Sephadex G-200 (results not shown), thus excluding the possibility that the loss of lectin activity was the result of dissociation. Impairment of the agglutinating activity by amino-group modification has been reported in the case of lentil (Vančurová *et al.*, 1976), pea (Trowbridge, 1973), *Ricinus* (Khan & Surolia, 1982), peanut (Nonnenmacher & Brossmer, 1981) and jack-fruit (Appukuttan & Basu, 1985) agglutinin. The presence of methyl α -D-galactopyranoside could not

prevent lysine group modification of the yeast lectin. The amino groups may be involved here in the maintenance of the proper conformation of the protein needed for its biological activity.

Modification of carboxy groups

Modification of carboxy groups by glycine ethyl ester resulted in the loss of agglutinating activity (Table 2), the presence of inhibitory sugar being partially protective against this. The number of carboxy groups modified may be determined by using radiolabelled glycine methyl ester (Hassing *et al.*, 1971). However, the number was not determined in the present case. From the inhibitory effect of glycine ethyl ester, the involvement of the carboxy groups in the maintenance of the proper structure, which is a prerequisite for the binding properties of the yeast lectin, cannot be ignored. Ionic interactions attributed to glutamic acid and aspartic acid residues have been reported to be a major criterion for hydrogen bonding with the hydroxy and acetamido groups of the sugar in the combining sites of various lectins (Goldstein & Hayes, 1978). Loss of agglutinating activity of potato lectin after carboxy-group modification has been demonstrated by Ashford *et al.* (1981). As with concanavalin A (Hassing *et al.*, 1971), the presence of the specific inhibitory sugar methyl α -D-galactopyranoside could partially protect against this modification. This could be explained either by a direct interaction between the carboxy groups of the lectin and galactoside in the binding sites, or by an altered conformational state of the lectin resulting from the association of the protein with the sugar. The effect of urea in decreasing the extent of inhibition of agglutination is very similar to that obtained by Ashford *et al.* (1981) with potato lectin. In the present case also, urea may bring about conformational changes in the lectin, preventing intramolecular amide-bond formation in the presence of carbodi-imide.

Modification of histidine residues

Ethoxyformic anhydride reacts primarily with the imidazole side chains at neutral pH (Melchior & Fahrney, 1970). It may react with thiol and amino groups, but at pH 7.0 the interaction with amino groups is unfavourable. To rule out the possibility of thiol-group modification the free thiol groups present after modification of histidine residues were quantified with 5,5'-dithiobis-(2-nitrobenzoic acid) and found to be comparable with those in the native lectin. Interestingly, modification of the first 15 histidine residues did not affect the agglutinating activity of the yeast lectin (Fig. 3), but an abrupt loss of activity was observed when residues 20–25 were modified (Fig. 4). However, this modification could not be prevented by the prior treatment of the lectin with inhibitory sugar, indicating the absence of a direct interaction of histidine residues with the sugar-binding site of the lectin. Jackson & Young (1986) have demonstrated a definite involvement of the imidazole ring of histidine in the restoration of the fully active conformational state of concanavalin A. Modification could be reversed by incubation with 1 M-hydroxylamine at pH 7 for 10 min, with restoration of agglutinating activity.

Modification of arginine residues

The guanido group of arginine was specifically modified with cyclohexane-1,2-dione. Blocking of 18

Fig. 1. Effect of succinic anhydride on the activity of yeast lectin

Succinylation was carried out as described in the Experimental section.

Fig. 2. Change in activity of succinylated yeast lectin as a function of blocked amino groups

Succinylation was carried out as described in the Experimental section.

Fig. 3. Effect of ethoxyformic anhydride on the activity of the yeast lectin

Ethoxyformylation was carried out as described in the Experimental section.

Fig. 4. Change in activity of the yeast lectin as a function of the number of histidine residues modified

Ethoxyformylation was carried out as described in the Experimental section.

arginine residues out of 30 led to loss of 75% of the agglutinating activity of the yeast lectin (Table 2). A similar extent of modification was also achieved in the presence of the inhibitory sugar. Very little is known about the role of arginine residues in protein structure and function. Arginine residues have been reported to be involved in protein-synthesis inhibition by ricin A-chain (Watanabe & Funatsu, 1986). Unblocking of the arginine residues by hydroxylamine was accompanied by almost complete restoration of the activity of the lectin.

Modification of thiol groups

Modification of thiol groups with 5,5'-dithiobis-(2-nitrobenzoic acid) or *N*-ethylmaleimide resulted in almost complete loss of agglutinating activity. Quantification of the free thiol groups with both these reagents, in the presence and the absence of the inhibitory sugar,

demonstrated that at least two thiol groups are present at the sugar-binding site (Table 2).

Immunodiffusion

All the modified lectin solutions showed a positive cross-reactivity with the native lectin (Fig. 5). These data suggest that chemical modification with group-specific modifying agents does not bring about any gross conformational change which may alter antigenicity of the yeast lectin. Similar results have been observed in the case of potato (Ashford *et al.*, 1981), *Ricinus* (Khan & Surolia, 1982) and *Abrus* agglutinin (Patanjali *et al.*, 1984).

The present study with the yeast lectin has revealed many interesting features. Like many other lectins, amino, carboxy and thiol (Roberts & Goldstein, 1984) groups have been found to be involved in the activity of the yeast lectin.

The role of amino, histidine and arginine residues in maintaining the conformation of the protein could be further investigated by c.d. studies. Unlike most lectins (Higuchi *et al.*, 1985; Privat *et al.*, 1976; Čermáková *et al.*, 1976), neither tryptophan nor tyrosine residues were involved in the activity of this lectin (Table 2). It

Fig. 5. Immunodiffusion tests of modified yeast lectin

Central well, anti-(yeast lectin); wells 1, 3, 5, 7, 9 and 11, native lectin; well 2, lysine residues modified by succinylation; well 4, carboxy groups modified; well 6, histidine residues modified; well 8, arginine residues modified; well 10, thiol groups modified; well 12, lysine residues modified by citraconylation.

appears that some of the active-site amino acids are common to those of other carbohydrate-binding proteins. On the other hand, however, this microbial lectin also possesses some unique characteristics.

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