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Binding site amino acid residues of jack fruit (*Artocarpus integrifolia*) seed lectin: Chemical modification and protein difference spectral studies

P. S. APPUKUTTAN* and DEBKUMAR BASU

Neurochemistry Division, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum 695 011, India

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Abstract. The effect of chemical modification of amino acid residues essential for sugar binding in the α-D-galactoside specific jack fruit (*Artocarpus integrifolia*) seed lectin and the protection of the residues by specific sugar from modification were studied. Citraconylation or maleylation of 75 % of its lysyl residues or acetylation of 70 % of the tyrosyl residues completely abolished sugar binding and agglutination without dissociation of subunits. 1-O-methyl α-D-galactoside could protect its essential lysyl and tyrosyl groups from modification. Tryptophan could not be detected in the protein. Difference absorption spectra on binding of the above sugar confirmed the role of tyrosine residues and showed an association constant $K = 0{\cdot}4 \times 10^3\ M^{-1}$. Data suggests that the lectin could be immobilized without any loss of sugar binding activity.

Keywords. Jack fruit seed lectin; chemical modification; difference spectra; binding site amino acids.

Introduction

Lectins are carbohydrate-binding and cell-agglutinating proteins of non-immune origin, widely distributed in plants and animals (Goldstein and Hayes, 1978; Barondes, 1981). Induction of mitogenic changes in resting lymphocytes (Naspitz and Ritcher, 1968) and preferential agglutination of transformed cells (Burger, 1971) are other properties exhibited by certain lectins. These proteins are therefore extensively used at present as ligands in affinity chromatography and structural analysis of glycoconjugates and in probing cell surface topology (Liener, 1976; Sharon and Lis, 1982). Lectins of varying specificity are at present isolated on a large scale from plant seeds. Purification and initial characterisation of an α-D-galactoside-binding lectin from the jack fruit (*Artocarpus integrifolia*) seeds have been reported from this laboratory (Sureshkumar *et al.,* 1982). The glycoprotein lectin of molecular weight 39,500 is a tetramer of identical subunits and possesses a very high specificity for the α-anomer of D-galactose. The high lectin content (1·3 % w/w) and availability in abundance of the seeds, combined with the ease of its purification enables extensive use of this lectin in glycoconjugate research.

* To whom correspondence should be addressed.

Abbreviations used: JSA, Jack fruit seed agglutinin; methyl α-gal, 1-O-methyl α-D-galactopyranoside; methyl α-glc, 1-O-methyl α-D-glucopyranoside; TNBS, trinitrobenzene sulphonate; PBS, 20 mM phosphate buffer with 150 mM NaCl, pH 7.4; SDS, sodium dodecyl sulphate.

Such applications require detailed knowledge of the amino acid residues of the lectin involved in its sugar binding function. In the present communication we describe chemical modification studies on jack fruit seed agglutinin (JSA) conducted for this purpose by observing (i) the effect of chemical modification of amino acid residues on lectin activity and (ii) the degree of protection from modification of these residues by bound sugar. Difference absorbance spectra of sugar-bound lectin was also measured to delineate the role of aromatic amino acids. While tryptophanyl residues were undetectable in the lectin, a fraction of its lysyl and tyrosyl residues were found essential for sugar binding.

Materials and methods

Chemicals

Citraconic anhydride, maleic anhydride, succinic anhydride, N-acetyl imidazole, 1-O-methyl α-D-galactopyranoside (methyl α-gal), 1-O-methyl α-D-glucopyranoside (methyl α-glc), and trinitrobenzene sulphonate (TNBS) were purchased from Sigma-Chemicals Company, St. Louis, Missouri, USA. Molecular weight standards were obtained from Pierce Chemicals Company, Rockford, Illinois, USA. All other chemicals used were of analytical grade and purchased locally.

Preparation of JSA

The lectin was prepared from locally available jack fruit seeds by affinity chromatography on cross linked guar gum by the method previously described by Sureshkumar *et al.* (1982).

Citraconylation and maleynation of amino groups

Lectin was citraconylated as described by Atassi and Habeeb (1972) with or without 0·2 M methyl α-gal or methyl α-glc using 400 μ1 reagent per mg protein. Maleylation was carried out essentially as described by Butler *et al.* (1969). A 3 mg/ml solution of JSA in 0·2 M sodium borate buffer pH 9·0 with and without methyl α-gal or methyl α-glc (0.2 M) was treated with maleic anhydride (3 mg/ml) at 25°C for 5 h. Excess reagent after both citraconylation and maleylation was removed by dialysis against 20 mM phosphate buffer with 150mM NaCl, pH 7·4 (PBS). Decitraconylation or demaleylation of the modified samples was effected by overnight dialysis against 0·2 M sodium acetate buffer, pH 3·9.

Affinity chromatography on cross-linked guar gum

Those citraconylated or maleylated protein samples that possessed hemagglutinating activity were applied on to a column (1 ml/mg protein) of cross-linked guar gum gel prepared as described earlier (Appukuttan *et al.,* 1977) and 1 ml fractions were collected. The column was washed with PBS and bound protein eluted by 0·1 M galactose was dialysed to remove the sugar.

Ultra-violet difference spectra

A Schimadzu double beam spectrophotometer with recorder and microprocessor was used. A 3 ml solution of lectin in PBS (6 mg/ml) was taken in both sample and reference cuvettes of 1 cm path length and the base line was recorded into the instrument's memory. Various amounts of 1 M solution of the ligand (methyl α-gal) in PBS were added to the sample cuvette and the spectra recorded at 25°C. The maximum volume added was limited to 20 μ1/ml so as to ignore volume change.

Acetylation with N-acetyl imidazole

A 3 mg/ml solution of JSA in 20 mM sodium barbitone buffer, pH 7·5 was acetylated by treating with 400 fold molar excess of N-acetyl imidazole for 1 h at 23°C, with and without 0·2 M methyl α-gal. The number of O-acetylated tyrosyl groups in the protein was calculated from the absorbance change at 278 nm on treatment with 1 M NH_2 OH measured in a Spectronic 21 spectrophotometer according to the method of Riordan *et al.* (1965). For selective acetylation of tyrosyl groups the amino groups in JSA were first blocked by citraconylation as above and after acetylation with N-acetyl imidazole, the amino groups were regenerated by decitraconylation. Total tyrosyl groups in the protein was determined after complete acetylation in the presence of 1 % sodium dodecyl sulphate (SDS).

Determination of free amino groups

Free amino groups in protein samples were determined using TNBS as described by Habeeb (1967) using bovine serum albumin as Standard which contains 61 free amino groups.

Determination of tryptophan residues

The tryptophan residues were determined by measuring the absorbance at 250 nm and 280 nm in a Spectronic 21 spectrophotometer before and after oxidation with a mixture of dimethyl sulphoxide, concentrated HCl and glacial acetic acid as described by Savige and Fontana (1977). Tryptophan was also measured using the spectral shift method of Bencze and Schmidt (1957).

Hemagglutination assay

Two fold serial dilutions of the protein samples (0·2 ml) were mixed with 5 % human group O erythrocyte suspension (0·05 ml) in the same buffer. The agglutination was scored after 2 h.

Protein determination and molecular weight

Protein in various samples was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Acetylated samples were assayed after reversal of O-acetylation by 1 M NH_2 OH. Molecular weights of protein samples were determined by gel filtration on a Bio-Gel P-100 column (2 cm × 80 cm) by the method of Andrews (1965) using bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome C as standards.

Results and discussion

Modification of amino groups

Anhydrides of the unsaturated dicarboxylic acids, like citraconic and maleic acids have been widely used for reversible acylation of free amino groups of proteins (Means and Feeney, 1971; Klapper and Klotz, 1972). The newly introduced carboxyl group, when protonated, destabilizes the acyl amide linkage, thus facilitating deacylation at acid pH values (Kirby and Lancaster, 1972). Unlike as in the case of saturated dicarboxylic acids, the free carboxyl group is held in orientation suitable for deacylation, by virtue of the carbon-carbon double bond.

Acylation of amino groups of JSA by citraconic or maleic anhydride completely destroys its hemagglutinating activity, modifying respectively 26 and 21 out of a total of 32 amino groups (table 1). Modification under identical conditions, but in the presence of the specific sugar, methyl α-gal (0·2 M) protects a fraction of the molecules from inactivation. During chromatography on the affinity adsorbent, cross-linked guar gum, about 25 % of the lectin dialysed after citraconylation or maleylation in the presence of methyl α-gal was found to retain its sugar affinity. The low degree of protection may be due to the unfavourable pH during acylation (8·2 and 9·0 respectively) and to prolonged exposure (4–5 h) to the reagents. During citraconylation and maleylation, bound sugar could protect a third of the modifiable amino groups as shown in the table. Agglutination capacities of the two acylated samples binding to guar gum and eluted

Table 1. Properties of JSA samples citraconylated or maleylated under various conditions.

Protein sample	Number of amino groups modified* (total = 32)	Minimum concentration (μg/ml) required for agglutination	Molecular weight
Native JSA	—	0·156	39,500
C-JSA	26	(N.A)	39,500
C-JSA (methyl α-Gal) binding to guar gum	16	0.312	(N.D)
C-JSA (methyl α-Gal) unbound to guar gum	26	(N.A)	40,000
C-JSA (methyl α-Glc)	25	(N.A)	(N.D)
M-JSA	21	(N.A)	38,000
M-JSA (methyl α-Gal) binding to guar gum	13	0.625	(N.D)
M-JSA (methyl α-Gal) unbound to guar gum	25	(N.A)	41,000
M-JSA (methyl α-Glc)	26	(N.A)	(N.D)

C-JSA and M-JSA are respectively the JSA samples citraconylated and maleylated as described under Methods. For C-JSA (sugar) and M-JSA (sugar), the protein was incubated with l-O-methyl-α-D-galactoside or 1-O-methyl α-D-glucoside (0·2 M) for 30' at 5°C before addition of modifying reagent.
N.A, Not agglutinating; N.D, not determined.
* Obtained from measurement of unmodified aminogroups.

with galactose were comparable to that of the unmodified lectin. These observations suggest that unmodified amino groups are essential at the sugar-binding site of the lectin and if provided this site is protected, modification of other amino groups does not affect sugar binding or agglutination. On the above basis the loss of sugar binding and agglutinability on citraconylation or maleylation does not appear to be due to conformational changes induced by modification of amino groups distant from the sugar binding site. The data also provides a method of immobilizing JSA for affinity chromatography through its amino groups using cyanogen bromide or glutaraldehyde.

The major fraction of modified lectin molecules fail to bind to cross-linked guar gum, despite the presence of 0·2 M (2600 molar excess) specific sugar during modification. The unbound sample is identical with the JSA modified without sugar, both in the extent of amino group modification and in the loss of agglutination. This observation along with the complete inability of the nonspecific methyl α-glc to protect the lectin against either modification (table 1) indicates that the protection offered by the specific sugar is not due to its non-specific interference in the reaction or consumption of the modifying reagent by the latter.

Acylation of amino groups by dicarboxylic acid anhydrides substitutes a positive charge by a negative charge, leading to drastic changes in electrostatic interactions and frequently to dissociation of oligomeric proteins (Klapper and Klotz, 1972; Shetty and Kinsella, 1980). JSA has been shown to be a tetramer of 4 identical 10,000 dalton subunits in noncovalent association. However, as shown in table 1, none of the modified inactivated protein samples differed from the native protein in molecular weight, as determined by Bio-Gel P-100 gel filtration. Subunit association in JSA probably involves amino groups which are therefore inaccessible to modification.

Maleic anhydride, the milder and slower of the two reagents, acylates a slightly lesser number of amino groups, as expected. Similar to most other proteins (Butler *et al.,* 1969; Khan and Surolia, 1982), deacylation of JSA leads to 75–100% recovery of agglutinating activity. Incubation with TNBS during determination of free amino groups has been shown not to affect the groups acylated by citraconic or maleic anhydride in contrast to acylation by dimethyl maleic anhydride (Butler *et al.,* 1969; Nieto and Palacian, 1983).

Role of tryptophan and tyrosine

The ultra-violet difference absorption spectrum of JSA on binding to methyl α-gal gave a positive maximum at 300 nm, a corresponding negative maximum at 255 nm and an isosbestic point at 280 nm (figure 1). The spectral difference is due to conformational changes resulting from sugar binding since methyl α-glc instead of methyl α-gal was ineffective. The magnitude of the positive difference absorption at 300 nm plotted as a function of ligand concentration (figure 2) was used to calculate the binding constant K of the ligand for JSA using the relation $K = [1/S]_{\text{free.}}$ Where $[S]_{\text{free}} = [S]$ total $- 0{\cdot}5\ n\ [P]$ and $[S]$ is the sugar concentration at 50 % saturation of lectin binding sites and $n\ [P]$ represents the lectin subunit concentration. The results indicate that JSA binds methyl α-gal with an association constant $K = 0{\cdot}4 \times 10^3\ M^{-1}$. The difference absorption spectra obtained in figure 1 is characteristic of the presence of typtophan and/or tyrosine at ligand binding site (Neurohr *et al.,* 1980). However, upon oxidation

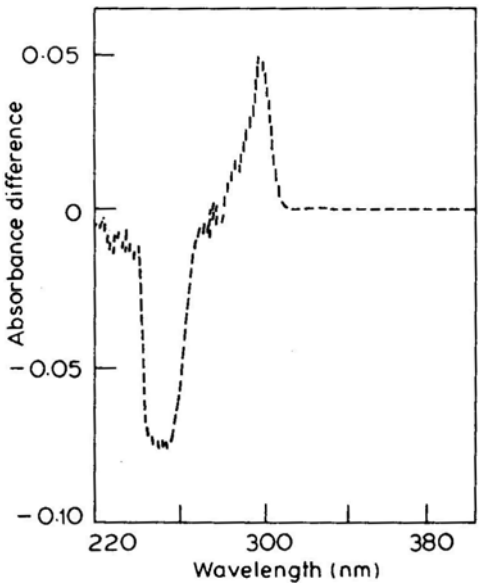

Figure 1. Ultra-violet difference spectra of JSA and methyl α-gal complex measured at 25°C, in PBS. Details are given under Methods.

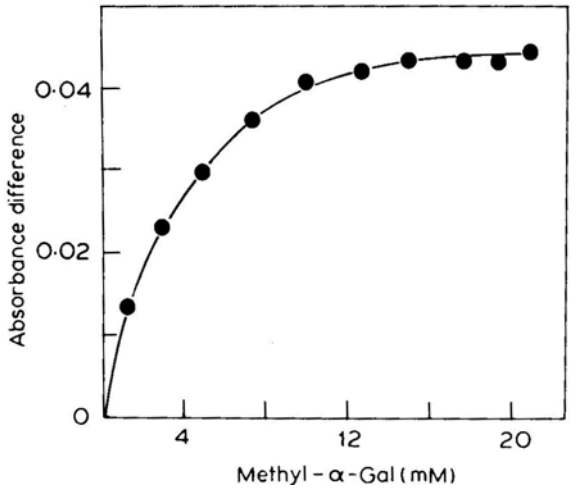

Figure 2. Variation of the magnitude of the maximum positive spectral difference as a function of the total ligand sugar (methyl *α*-gal) concentration measured at 25°C in PBS.

of the protein with dimethyl sulphoxide/HCl/acetic acid mixture the decrease in absorbance at 280 nm was not accompanied by an increase in absorbance at 250 nm, indicating the absence of tryptophan residues (Savige and Fontana, 1977). Tryptophan could not be detected by spectral shift method either. Modification of tryptophan

residues with N-bromosuccinimide was not attempted since this reagent is known to modify tyrosyl and methionyl groups also (Spande and Witkop, 1967).

Modification of tyrosyl residues

Tyrosyl residues form part of the sugar binding site of JSA since selective as well as non-selective acetylation of these residues by N-acetyl imidazole completely abolished its activity, modifying 12 tyrosyl groups in both the cases (table 2). Incubation with 0·2 M methyl α-gal could protect half of these tyrosyl groups as well as retain most of its hemagglutinating activity. Denaturing with 1 % SDS exposes 5 more tyrosyl groups of the protein for acetylation, indicating that at least one tyrosyl group in each of the 4 subunits of the protein molecule is masked during subunit assembly.

Table 2. Effect of acetylation with N-acetyl imidazole under various conditions, on tyrosyl residues and hemagglutinating activity of JSA. Details are given under Methods.

Protein sample	Number of tyrosyl residues modified	Minimum concentration (μg/ml) required for agglutination
Native JSA	—	0·156
JSA acetylated without SDS	12	(N.A)
JSA acetylated with 1% SDS	17	(N.A)
JSA acetylated with 0·2 M methyl α-gal	6	5·0
JSA acetylated selectively at tyrosyl residues	12	(N.A)

N.A, No agglutination.

Amino acid residues that cannot be modified without affecting sugar affinity are not the same even among D-galactoside specific lectins. Thus lysyl and tyrosyl group modification negligibly affected the activities of peanut and abrus agglutinin respectively (Nonnenmacher and Brossmer, 1981; Patanjali *et al.,* 1984). The latter also requires its tryptophanyl residues intact for activity. *Ricinus communis* agglutinin activity on the other hand is completely destroyed on modification of its amino and tyrosyl residues, but not tryptophan residues (Khan and Surolia, 1982). JSA as indicated by the present data resembles the latter lectin possessing a relatively more polar sugar-binding site.

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