Four identical subunits in jack fruit seed agglutinin offer only two saccharide binding sites

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Gel filtration of jack fruit seed agglutinin in 6 M guanidine hydrochloride confirmed our earlier report that the native 39.5-kDa protein was a tetramer of identical noncovalently associated 10-kDa subunits. Binding studies by the fluorescence quenching method using 4-methylumbelliferyl α-D-galactoside as well as equilibrium dialysis using *p*-nitrophenyl α-D-galactoside indicated only two binding sites per tetramer. This behaviour resembles the half-of-the-sites reactivity in certain enzymes and is discussed in view of the small subunit size.

Jack fruit seed agglutinin *Half-of-the-sites binding* *Equilibrium dialysis* *Fluorescence quenching*

1. INTRODUCTION

A lectin with marked specificity for α-linked galactosides had been isolated from jack fruit seed *(Artocarpus integrifolia)* and the initial characterisation reported [1]. The native 39.5-kDa glycoprotein was shown by SDS gel electrophoresis to consist of 4 identical 10-kDa subunits in non-covalent association. While tryptophan residues could not be detected, part of the lysyl and tyrosyl groups of the lectin was found to be essential for carbohydrate binding [2]: in this communication, after confirming the subunit molecular mass by gel filtration in 6 M Gn.HCl the sugar binding stoichiometry of the lectin was examined by two different methods: titration of quenching of a fluorescent sugar derivative on binding to lectin and equilibrium dialysis. The results indicate that the homotetrameric lectin offers only two sugar binding sites and is unique among lectins in this respect.

Abbreviations: JSA, jack fruit seed agglutinin; PBS, potassium phosphate buffer (20 mM, pH 7.4) containing 150 mM NaCl; MeUmb α-Gal, 4-methylumbelliferyl α-D-galactopyranoside; PNP α-Gal, *p*-nitrophenyl α-D-galactopyranoside; Gn.HCl, guanidine hydrochloride

2. MATERIALS AND METHODS

Jack fruit seed agglutinin (JSA) was isolated from local seeds as in [1]. PNP α-Gal, MeUmb α-Gal and *N*-ethylmaleimide were purchased from Sigma, USA. Gn.HCl was purchased from Riedel-De Haen, Hannover, FRG.

The molecular mass of JSA subunit was determined by gel filtration according to the method of Andrews [5] on Sepharose 6B in 6 M Gn.HCl using cytochrome *c*, chymotrypsinogen, ovalbumin, bovine serum albumin, concanavalin A [3] and winged bean agglutinin [4] as standards.

The binding of MeUmb α-Gal to JSA was monitored by measuring the quenching of fluorescence of the sugar in a Perkin Elmer MPF-44A ratio recording spectrofluorimeter in PBS solution. The samples were in 1 × 1 × 4 cm cuvettes mounted on thermostatically controlled holders. To 1 ml of 3 μM fluorescent sugar were added varying quantities of 160 μM JSA and the fluorescence spectra after each addition were recorded above 330 nm at 25°C. In a separate experiment, to a fixed amount of protein (1 ml, 25 μM) were added various aliquots of a 300 μM solution of MeUmb α-Gal, the fluorescence maximum

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measured at 375 nm and corrections made for dilution. This titration was done at 5 and 25°C. Equilibrium dialysis was carried out in 0.9 ml perspex cells separated by a semipermeable membrane made from dialysis tubing, using 3.5 mg/ml JSA and varying concentrations of PNP α-Gal in PBS. Equilibrium was allowed for 48 h at 5°C and free PNP α-Gal measured at 313 nm. The data were plotted according to Scatchard [5].

For modifying sulfhydryl groups, JSA was treated with a 10-fold molar excess of *N*-ethylmaleimide at 25°C in 20 mM potassium phosphate buffer, pH 7.0 containing 150 mM NaCl. Excess reagent was removed by dialysis and agglutinability towards human group O erythrocytes measured as in [4].

3. RESULTS

JSA was found to contain a single type of subunit of 10 kDa by gel filtration in 6 M Gn.HCl. The fluorescence of MeUmb α-Gal was progressively quenched with successive additions of JSA at 25°C (fig.1A). The fluorescence spectra recorded a maximum at 375 nm. With a large excess of protein the fluorescence tended to zero, showing that the fluorescence of lectin-bound ligand was negligible so that the value observed after successive additions of MeUmb α-Gal to a fixed amount of lectin was a measure of the free ligand. As shown in fig.1B, the Scatchard curve for binding at 5°C was steeper than that at 25°C, indicating a much faster binding at the lower tem-

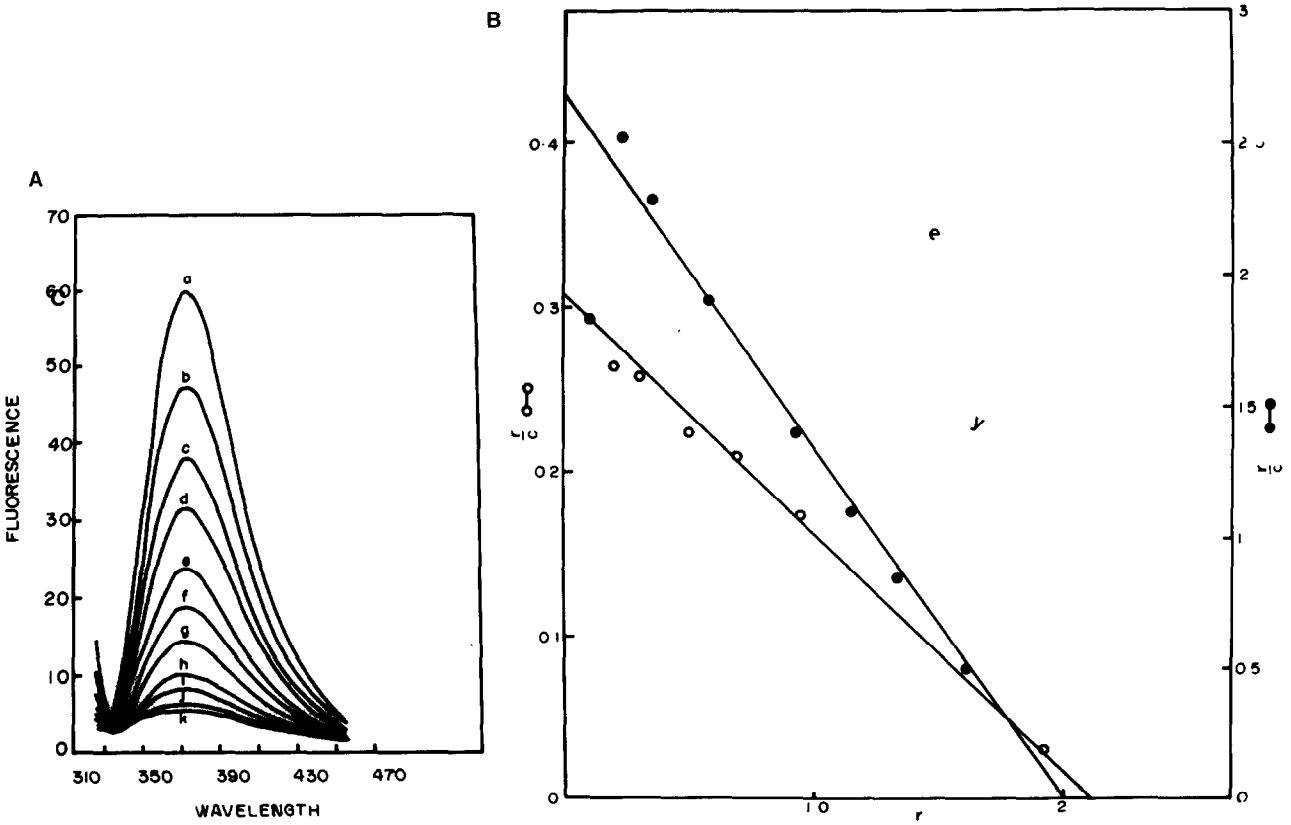

Fig.1. Quenching of fluorescence of MeUmb α-Gal by JSA in PBS medium after excitation at 318 nm. (A) Quenching of fluorescence on addition of increasing quantities of lectin at 25°C. To 1 ml of 3 μM solution of MeUmb α-Gal in a 1 × 1 × 4 cm cuvette, the following aliquots of 160 μM JSA were added: (a) 0 μl, (b) 10 μl, (c) 20 μl, (d) 30 μl, (e) 50 μl, (f) 70 μl, (g) 100 μl, (h) 150 μl, (i) 200 μl, (j) 300 μl, (k) 400 μl. (B) Scatchard plot for the binding of MeUmb α-Gal to JSA at 25°C (○—○) and 5°C (●—●) conducted as described in section 2. Residual fluorescence at 375 nm after each addition corresponding to free MeUmb α-Gal was noted.

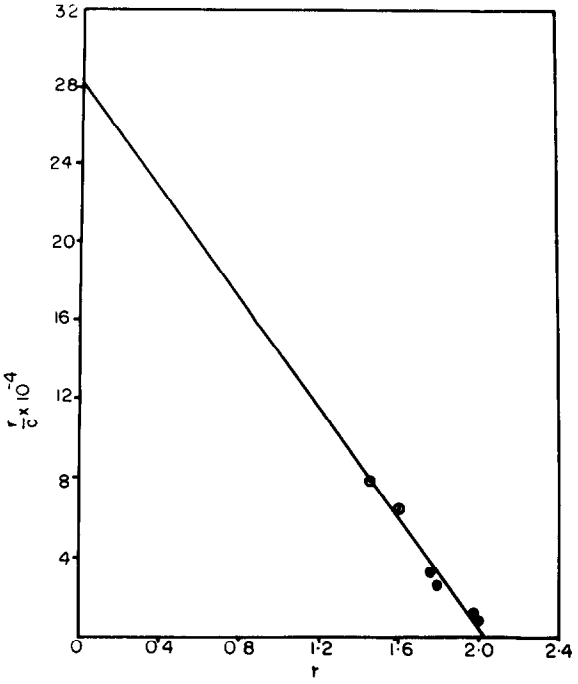

Fig.2. Scatchard plot of the binding of PNP α-D Gal to JSA. Equilibrium was attained in PBS at 5°C for 48 h using 3.5 mg/ml JSA. For details see text.

perature. The association constants calculated were 13.4×10^5 and 2.14×10^5 M^{-1} at 5 and 25°C, respectively. However, the number of binding sites per 39.5-kDa lectin was 2 at both temperatures. A Scatchard plot of equilibrium dialysis data using PNP α-Gal also showed two sugar-binding sites per 39.5-kDa lectin, with an association constant of 1.4×10^5 M^{-1} (fig.2). Treatment with *N*-ethylmaleimide did not affect the hemagglutinating activity of the lectin.

4. DISCUSSION

The gel filtration results show that JSA contains identical subunits of 10 kDa, in close agreement with our earlier reported value of 10.5 kDa, obtained by SDS-PAGE [1]. The native 39.5-kDa protein therefore should be a tetramer. However, the results of the fluorescence quenching titration as well as equilibrium dialysis given above clearly show only 2 binding sites per 39.5-kDa protein. This behaviour of identical subunits offering only half as many sugar binding sites has not been observed in any other lectin.

Soybean agglutinin which was initially reported to contain 4 identical subunits and two sugar-binding sites [6] was later shown by the same laboratory to contain dissimilar subunits [7] and 4 binding sites [8]. Goldstein and his co-workers could measure only two *N*-acetyl-D-galactosamine binding sites on the homotetrameric lectin (component III) from lima bean [9]. However, the same authors recently observed that the decreased sugar binding was due to oxidation of essential thiol groups. The lectin prepared under non-oxidative conditions retained one binding site on each subunit [10]. In JSA, however, thiol groups could not be detected [1] nor did treatment with *N*-ethylmaleimide as described above cause any reduction in hemagglutinating activity.

The anomaly of a single substrate binding site per two identical subunits has been noted in enzymes such as yeast [11] and *E. coli* [12] UDP-glucose 4-epimerases, yeast glyceraldehyde-3-phosphate dehydrogenase [13] and liver alcohol dehydrogenase [14]. Such 'half-of-the-sites reactivity' in enzymes may result from artifacts such as the failure to remove substrates bound prior to stoichiometry study or from the presence of a proportion of inactivated enzyme. However, dialysis of JSA has been found to dissociate completely even such sugars as MeUmb α-Gal or PNP α-Gal which bind many times stronger than the galactose used for elution of the lectin during isolation. Moreover, only chromatographically homogeneous protein was used in the two binding studies. The monophasic Scatchard plot obtained in both cases also rules out a negative co-operative effect.

An alternative explanation is that a single binding site is constituted jointly by two subunits, as proposed in the case of some enzymes [15]. The reason might be the unusually small size (10 kDa) of each subunit that cannot fully accommodate one saccharide unit. Data are lacking on the sugar binding stoichiometry of an L-fucose binding lectin containing 12 subunits, each of M_r 10000 isolated from the eel serum *(Anguilla anguilla)* [16]. Elucidation of the minimum molecular size of the saccharide binding site in galactose-specific lectins may verify the above proposition.

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