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## THE USE OF 4-METHYLUMBELLIFERYL GLYCOSIDES IN BINDING STUDIES WITH THE LECTINS BS I-A<sub>4</sub>, BS I-B<sub>4</sub> AND BS II FROM *BANDEIRAEA (GRIFFONIA) SIMPLICIFOLIA*

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### 1. Introduction

Seeds of *Bandeiraea simplicifolia* (*Griffonia simplicifolia*) contain 12 lectins and isolectins distributed over the 4 groups BS I–IV [1]. This paper deals with the tetrameric and tetravalent lectins BS I and BS II [2]. BS II is a GlcNAc-specific lectin [3,4]. The BS I group is composed of 5 Gal-binding isolectins A<sub>4</sub>, A<sub>3</sub>B, A<sub>2</sub>B<sub>2</sub>, AB<sub>3</sub> and B<sub>4</sub> with degrees of A or B blood-group specificity determined by the ratio of subunit A (GalNAc specific) over subunit B (Gal specific) [5–8]. The degree of  $\alpha$ - or  $\beta$ -anomeric binding preference is influenced by the nature of the aglycon; this forms the basis of a reproducible separation of the 5 BS I isolectins by affinity chromatography using aryl  $\beta$ -glycosides as ligand arms [9]. The ligands used here for binding studies in solution are 4-methylumbelliferyl-(MeUmb-) glycosides. Their versatility is apparent from:

- (i) The fluorescence of MeUmb  $\alpha$ -Galp, MeUmb  $\beta$ -Galp and MeUmb  $\beta$ -GalNAcp that is quenched totally upon binding with BS I-B<sub>4</sub> or almost totally with BS I-A<sub>4</sub>;
- (ii) The discriminative difference absorption spectra

of the MeUmb group when MeUmb  $\alpha$ -Galp binds to BS I-A<sub>4</sub> or to BS I-B<sub>4</sub>;

- (iii) The results of titration of the increase in MeUmb-fluorescence polarisation [10,11] for a series of MeUmb  $\beta$ (GlcNAcp)<sub>n</sub> glycosides as ligands for BS II.

### 2. Materials and methods

BS I-A<sub>4</sub>, BS I-B<sub>4</sub> and BS II were obtained as in [9]. BS I-A<sub>4</sub> and BS I-B<sub>4</sub> were dissolved in and dialyzed against 0.05 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 0.15 M NaCl, 1 mM CaCl<sub>2</sub> (pH 7.2). For BS II, the same buffer also contained 0.1 mM MnCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>. Concentrations of lectins were determined with  $\epsilon_{280} = 1.41 \text{ cm}^2/\text{mg}$  for BS I [5] and  $1.03 \text{ cm}^2/\text{mg}$  for BS II [12] and were expressed as binding sites on the basis of  $M_r = 28\,500$  for a subunit A and B of BS I [5] and of BS II [12]. The fluorescent ligands were MeUmb  $\beta$ (GlcNAcp)<sub>n</sub>, with  $n = 1, 2$  or  $3$  [13,14], MeUmb  $\beta$ (GlcNAcp)<sub>4</sub>, a gift from Professor Kozo Hamaguchi [15], MeUmb  $\alpha$ -Galp, MeUmb  $\beta$ -Galp and MeUmb  $\beta$ -GalNAcp (Koch-Light). These and other carbohydrates (Koch-Light) were dissolved in the appropriate buffer.

Difference absorption spectra were obtained with thermostatted  $2 \times 0.437 \text{ cm}$  tandem mixing cuvettes in a Zeiss PMQII-M4QIII apparatus [16]. Fluorescence measurements were made with an Aminco SPF-500 ratio instrument and spectra were uncorrected. Excitation of MeUmb-glycoside fluorescence was at 325 nm and emission was measured at 373 nm. A ligand solution ( $0.9\text{--}2.9 \mu\text{M}$ ,  $604 \mu\text{l}$ ) was contained in a thermostatted  $0.4 \times 1 \times 4.5 \text{ cm}$  cuvette. It was titrated by adding a maximal total volume of  $130 \mu\text{l}$

**Abbreviations:**  $M_r$ , relative molecular mass; GalNAc, 2-acetamido-2-deoxy-D-galactose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; Me  $\alpha$ -Galp, methyl  $\alpha$ -D-galactopyranoside; MeUmb  $\alpha$ -Galp, 4-methylumbelliferyl  $\alpha$ -D-galactopyranoside; MeUmb  $\beta$ -Galp, 4-methylumbelliferyl  $\beta$ -D-galactopyranoside; MeUmb  $\beta$ -GalNAcp, 4-methylumbelliferyl  $\beta$ -2-acetamido-2-deoxy-D-galactopyranoside; MeUmb  $\beta$ -GlcNAcp, 4-methylumbelliferyl  $\beta$ -2-acetamido-2-deoxy-D-glucopyranoside; MeUmb  $\beta$ (GlcNAcp)<sub>2</sub>, 4-methylumbelliferyl-*N,N'*-diacetyl- $\beta$ -chitobioside; MeUmb  $\beta$ (GlcNAcp)<sub>3</sub>, 4-methylumbelliferyl-*N,N',N''*-triacyl- $\beta$ -chitotrioside; MeUmb  $\beta$ (GlcNAcp)<sub>4</sub>, 4-methylumbelliferyl-*N,N',N'',N'''*-tetraacyl- $\beta$ -chitotetraoside

concentrated protein solution in successive portions with intermediate mixing. All data were corrected for a blank titration without MeUmb glycoside, using the corresponding protein at each temperature. The dilution-corrected titration curves of MeUmb fluorescence and MeUmb-fluorescence polarisation were linearized [17], without approximation concerning the concentration of free protein sites.

### 3. Results and discussion

Upon binding of GlcNAc to BS II, protein fluorescence with  $\lambda_{\max} = 329$  nm decreases drastically with a shift of  $\lambda_{\max}$  to 334 nm. In contrast, protein fluorescence of BS I-A<sub>4</sub> ( $\lambda_{\max} \sim 325$  nm) and especially that of BS I-B<sub>4</sub> ( $\lambda_{\max} 327$  nm) increases upon binding with carbohydrates. With BS I-A<sub>4</sub>, this increase is very small and is accompanied by a blue shift as observed with Me  $\alpha$ -Galp and with GalNAc. The increase in BS I-B<sub>4</sub> fluorescence, caused by Me  $\alpha$ -Galp or by melibiose does not affect the position of the emission maximum.

Many of the above effects, in particular those for BS I-B<sub>4</sub> and especially for BS I-A<sub>4</sub>, seemed somewhat small for reliable quantitation of carbohydrate binding. The use of MeUmb glycosides was therefore tested. All effects observed with these ligands (fluorescence quenching, increase in fluorescence polarisation and difference absorption spectra) were abolished by saturating amounts of the corresponding reducing sugar or Me-glycoside.

It was found that the fluorescence of MeUmb  $\alpha$ -Galp, MeUmb  $\beta$ -Galp and MeUmb  $\beta$ -GalNAcp is totally quenched upon binding to BS I-B<sub>4</sub> or is quenched to a large extent and in a temperature-dependent way upon binding to BS I-A<sub>4</sub>; this allows sensitive evaluation of the association constants  $K$ , e.g., at  $\sim 13.5^\circ\text{C}$  (fig.1) and at  $25^\circ\text{C}$  (table 1). From these data the binding enthalpy,  $-\Delta H^\circ$ , was estimated to be 15–22 kJ/mol for MeUmb  $\beta$ -GalNAcp or MeUmb  $\alpha$ -Galp with BS I-A<sub>4</sub> and 43–49 kJ/mol for MeUmb  $\alpha$ -Galp or MeUmb  $\beta$ -Galp with BS I-B<sub>4</sub>.

Next to the different carbohydrate specificities of the A and B subunits, the above differences in tryptophane fluorescence upon binding of carbohydrates, in MeUmb-fluorescence quenching and in  $\Delta H^\circ$  for MeUmb glycoside binding, observed with BS I-A<sub>4</sub> and BS I-B<sub>4</sub>, point at different ligand interactions for both homo-isolectins. This can be due in part to the aryl aglycon

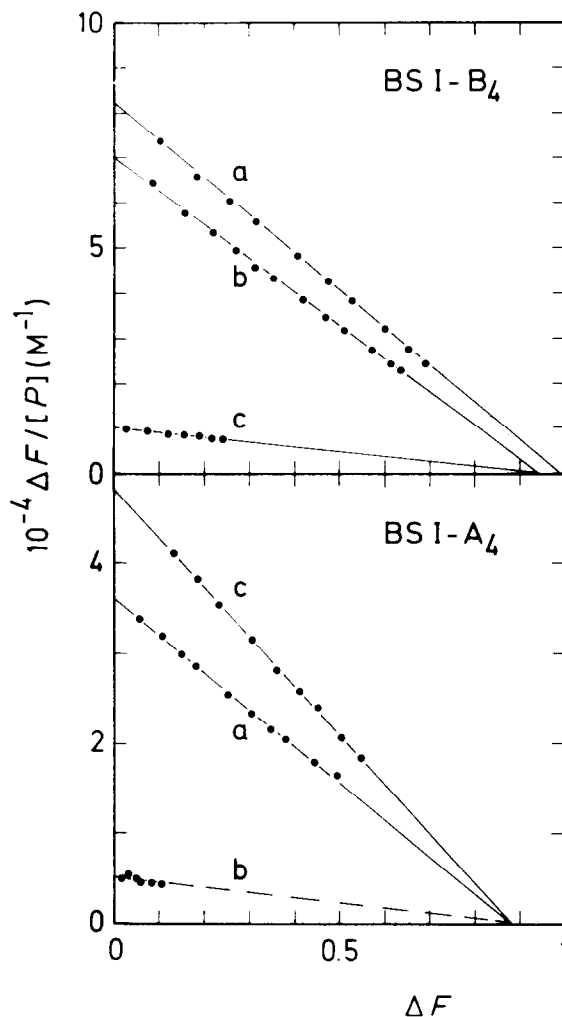


Fig.1. Linearized [15] titration curves of ligand-fluorescence quenching of  $0.92 \mu\text{M}$  MeUmb  $\alpha$ -Galp (a),  $1.63 \mu\text{M}$  MeUmb  $\beta$ -Galp (b) and  $1.47 \mu\text{M}$  MeUmb  $\beta$ -GalNAcp (c) by an excess ( $\leq 30 \mu\text{M}$ ) of the homo-isolectins BS I-B<sub>4</sub> at  $13.1^\circ\text{C}$  (top) and BS I-A<sub>4</sub> at  $13.7^\circ\text{C}$  (bottom).  $\Delta F$  is the relative fluorescence quenching and  $[P]$  is the concentration of free sites; both are corrected for dilution. A 1:1 complex was assumed to calculate  $[P]$  starting with a value of  $K$  corresponding to the slope of a  $\Delta F/[P_0]$  vs  $\Delta F$  plot,  $[P_0]$  being the concentration of free plus occupied sites. As obtained by iteration, the slope increased to a constant value as illustrated. The values of  $K$  (in  $\text{M}^{-1}$ ) and  $\Delta F_\infty$ , at infinite  $[P]$ , were calculated from the slope and intercept. For BS I-B<sub>4</sub>:  $(8.33 \pm 0.05)10^4$  and  $0.99 \pm 0.01$  with MeUmb  $\alpha$ -Galp;  $(7.47 \pm 0.08)10^4$  and  $0.94 \pm 0.01$  with MeUmb  $\beta$ -Galp;  $(1.04 \pm 0.08)10^4$  and  $0.96 \pm 0.07$  with MeUmb  $\beta$ -GalNAcp. For BS I-A<sub>4</sub>:  $(5.45 \pm 0.07)10^4$  and  $0.885 \pm 0.015$  with MeUmb  $\beta$ -GalNAcp;  $(4.04 \pm 0.07)10^4$  and  $0.89 \pm 0.02$  with MeUmb  $\alpha$ -Galp;  $\sim 5.6 \times 10^3$  and consistent with  $\Delta F_\infty = 0.9$  with MeUmb  $\beta$ -Galp.

Table 1  
Binding characteristics of MeUmb-glycosides and the two  
BS I homo-isoelectins at 25°C

	$\Delta F_{\infty}$	$10^{-4} \times K$
BS I-A <sub>4</sub>		
MeUmb $\alpha$ -Galp	$0.77 \pm 0.03$	$2.8 \pm 0.1$
MeUmb $\beta$ -GalNAcp	$0.79 \pm 0.02$	$4.3 \pm 0.1$
MeUmb $\beta$ -Galp	a	a
BS I-B <sub>4</sub>		
MeUmb $\alpha$ -Galp	$0.99 \pm 0.02$	$4.1 \pm 0.1$
MeUmb $\beta$ -GalNAcp	b	b
MeUmb $\beta$ -Galp	$0.98 \pm 0.05$	$3.3 \pm 0.1$

<sup>a</sup> The data at 13.7°C are consistent with  $\Delta F_{\infty} = 0.90$  and  $K = 1.0 \times 10^4 \text{ M}^{-1}$  (fig.1)

<sup>b</sup> At 13.1°C these values are 0.96 and  $5.6 \times 10^3 \text{ M}^{-1}$  (fig.1)

The data were obtained as in fig.1; see text for  $\Delta H^{\circ}$  values

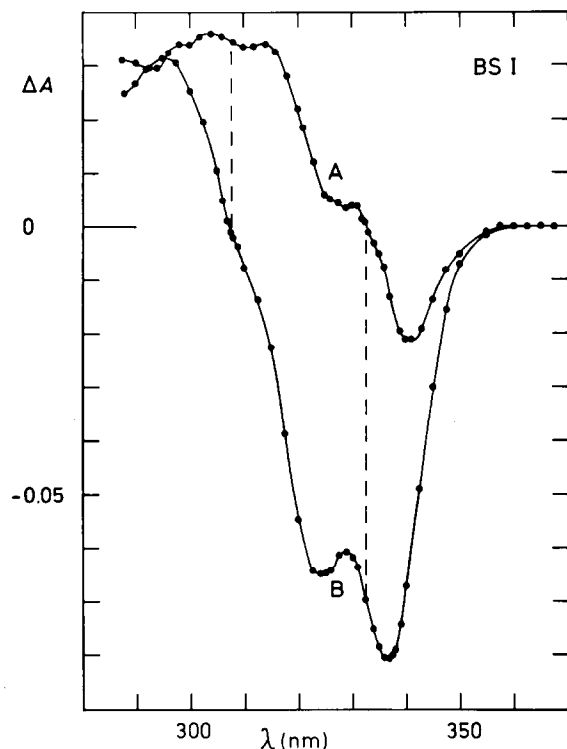


Fig.2. Difference absorption spectra obtained at 13°C with 93  $\mu\text{M}$  MeUmb  $\alpha$ -Galp using either 69  $\mu\text{M}$  BS I-A<sub>4</sub> ((A) 45  $\mu\text{M}$  complex) or 91  $\mu\text{M}$  BS I-B<sub>4</sub> ((B) 64  $\mu\text{M}$  complex). When measured at the extreme absorption differences, the MeUmb-difference spectra disappeared in the presence of 40 mM  $\alpha$ -Galp. The dashed vertical lines indicate the wavelengths suggested to monitor binding by the A and B subunits in the hetero-isoelectins of BS I.

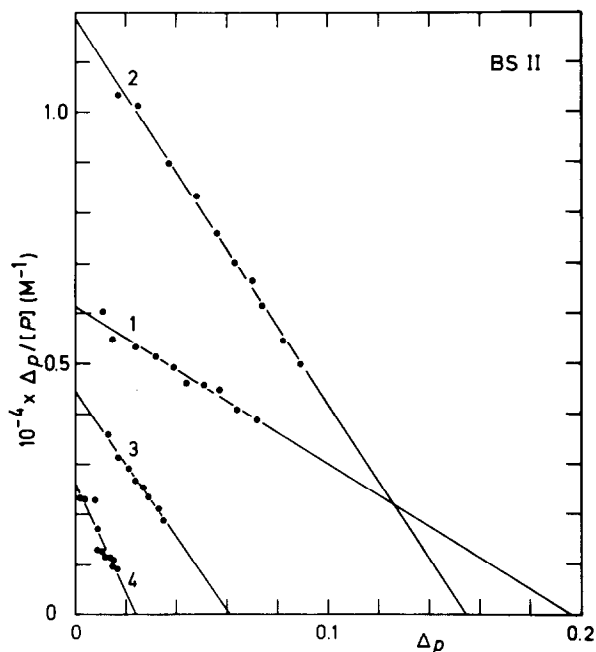


Fig.3. Linearized [15] titration curves of the increase in ligand-fluorescence polarisation of 2.5–2.9  $\mu\text{M}$  MeUmb  $\beta$ (GlcNAcp)<sub>n</sub> by an excess ( $\leq 20 \mu\text{M}$ ) of BS II at  $\sim 13.5^\circ\text{C}$ .  $n$  (= 1, 2, 3 or 4 as indicated) is the number of GlcNAc residues in the ligands,  $\Delta p$  is the increase in fluorescence polarisation and  $[P]$  was calculated as in fig.1. As checked for the most intense signal changes at the end of the titrations with  $n = 1$  and  $n = 2$ , the initial values of  $p$  for free ligand (0.167 for  $n = 1$  and 0.223 for  $n = 2$ ) were restored upon addition of a large excess of GlcNAc when corrected for the protein blank; the initial values were  $p = 0.250$  for  $n = 3$  and  $p = 0.251$  for  $n = 4$ . The values of  $K$  (slope) and  $\Delta p_{\infty}$  (intercept on  $\Delta p$  axis) are gathered in table 2.

since the ratio of  $K$  values of the MeUmb glycosides and those of the corresponding  $p$ -NO<sub>2</sub>Phe glycosides [6,8] are practically identical for BS I-A<sub>4</sub> but are different for BS I-B<sub>4</sub>. Indeed, a different surrounding of the MeUmb group is very apparent from the difference absorption spectra when MeUmb  $\alpha$ -Galp binds to BS I-A<sub>4</sub> or to BS I-B<sub>4</sub> (fig.2). With BS I-B<sub>4</sub> it arises from a blue shift and shows two minima at 336.5 nm and 325 nm with an isosbestic point at 307 nm which is very similar to the difference absorption spectrum for 4-methylumbelliferyl  $\alpha$ -D-mannopyranoside and concanavalin A [16]. With BS I-A<sub>4</sub> the difference absorption spectrum shows a minimum at 340 nm, an isosbestic point at 333 nm and is maximal between 300 and 315 nm. It can be expected that measure-

Table 2

Association constants and increase in MeUmb-fluorescence polarisation for binding of 4 MeUmb  $\beta$ (GlcNAc) $_n$  glycosides to BS II

No. of GlcNAc	$10^{-4} \times K$ (M $^{-1}$ )	$\Delta p_{\infty}$
$n = 1^a$	$3.1 \pm 0.2$	$0.196 \pm 0.012$
$n = 2^a$	$7.7 \pm 0.2$	$0.154 \pm 0.004$
$n = 3^b$	$7.3 \pm 0.3$	$0.061 \pm 0.003$
$n = 4^b$	$10.8 \pm 1.4$	$0.024 \pm 0.004$

$a 14.3^{\circ}\text{C}$ ;  $b 13.5^{\circ}\text{C}$

The values were obtained by linear regression of the data in fig.3

ments at 333 nm and 307 nm should selectively monitor binding by the B and A subunit in BS I hetero-isolectins; this aspect will be treated elsewhere.

Upon binding to BS II, a positive absorption difference spectrum was observed with MeUmb  $\beta$ -GlcNAc (not shown); except for its sign, it was similar to the one for the system MeUmb  $\alpha$ -Galp  $\cdot$  BS I-B $_4$  (fig.2). When fluorescence of a MeUmb  $\beta$ (GlcNAc) $_n$  glycoside was measured in the presence of BS II, quenching was very small, if any. This contrasts with the observed fluorescence changes upon binding to wheat germ agglutinin [14,18,19], to lysozyme [13,15,17] and to GlcNAc-directed antibodies [20]. However, binding of MeUmb  $\beta$ (GlcNAc) $_n$  to BS II could be quantitated by the increase in MeUmb-fluorescence polarisation,  $\Delta p$ , as a measure of the decreased motility of the MeUmb group in a complex. As shown by the data in fig.3 and table 2,  $K$  for MeUmb  $\beta$ (GlcNAc) $_n$  increases and tends to become constant for  $n \geq 2$ . This is consistent with the data for the parent chito-oligosaccharides [3,4]. Moreover, the increase in MeUmb-fluorescence polarisation upon formation of a MeUmb  $\beta$ (GlcNAc) $_n \cdot$  BS II complex ( $\Delta p_{\infty}$ ) decreases as a function of  $n$  (table 2). These data, together with the observation that B-D-Gal-(1 $\rightarrow$ 4)-D-GlcNAc is an extremely poor ligand, suggest that the carbohydrate-binding site of BS II should accommodate a chitobioside structure through the non-reducing end in a chito-oligosaccharide.

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