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Lectins from *Griffonia simplicifolia* seeds★

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Abstract. The physical-chemical and carbohydrate binding specificity of *Griffonia simplicifolia* I (GS I) isolectins, one of the 4 lectins isolated from *Griffonia simplicifolia* seeds, are described.

Association constants for the binding of methyl α- and β-D-galactopyranoside and methyl 2-acetamido-2-deoxy-α-D-galactopyranoside to the A_4, A_2 B_2 and B_4 isolectins are reported.

Precipitation reactions of the *Griffonia simplicifolia* isolectins with guaran and type B blood group substance are described.

The hypothesis that subunit B is a precursor of subunit A, a process involving proteolytic cleavage of the B subunit, was tested by conducting structural studies on the 2 subunits. The results indicated that the A and B subunits are probably products of 2 separate but closely related, possibly contiguous genes.

Keywords. Lectin; *Bandeiraea simplicifolia; Griffonia simplicifolia;* isolectins; carbohydrate binding.

Introduction

Griffonia simplicifolia, formerly called *Bandeiraea simplicifolia,* is a woody shrub which grows in the rain forests of Northwest Africa. It is especially abundant in Ghana. The mature plant reaches a height of 3–4 metres and develops pods which explode with great force dispersing the flat, round black seeds. Thus far we have identified and isolated four distinctly different lectins from *G. simplicifolia* seeds:

GS I—A system of 5 α-D-galactosyl binding isolectins
GS II—An N-acetyl-D-glucosaminyl binding lectin
GS III—A system of 5 2-acetomido-2-deoxy-α-D-galactosyl binding isolectins
GS IV—A lectin with Le^b (Lewis b) binding properties

This report will focus on the GS I isolectins.

Results

Mäkela and Mäkela (1956) first reported a human anti-B haemagglutinating activity in *G. simpliafolia* seeds. Employing an affinity matrix prepared by copolymerization of acrylamide with allyl α-D-galactoside, Horejsi and Kocourek (1973)

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Abbreviations used: GS I, A system of 5α-D-galactosyl binding isolectins; GS II, a N-acetyl-D-glucosaminyl binding lectin, GS III, a system of 5,2-acetamido-2-deoxy-α-D-galactosyl binding lectins; GS IV, a lectin with Le^b (Lewis-b) binding properties, all lectins isolated from *G. simplicifolia* seeds; PAGE, polyacrylamide gel electrophoresis; SDS-AGE, sodium dodecylsulphate PAGE; BSA, bovine serum alubmin; *p*-NO_2 - ph, *p*-nitrophenyl.

purified the anti-blood group B lectin but did not characterize it. Subsequently, Hayes and Goldstein (1974) obtained what they believed to be a homogeneous glycoprotein by affinity chromatography on a melibionate Bio-Gel column. The glycoprotein gave one band on polyacrylamide gel electrophoresis (PAGE) at pH 4.3, one band on sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) at pH 7.0, a single symmetrical peak in the ultra-centrifuge and was quantitatively precipitated by the galactomannan guaran. However, multiple bands were observed on PAGE at pH 9.5 and on isoelectric focusing. A subunit molecular weight of 31,000 was calculated from gel electrophoresis, and 28,500 from the amino acid and carbohydrate composition. The intact lectin is composed of 4 subunits and has a molecular weight of approximately 114,000, as determined by sedimentation velocity centrifugation and gel filtration. A metalloprotein, the lectin requires Ca^{2+} for maximum activity. Analysis of the lectin by circular dichroism spectroscopy indicated that the native lectin contains a large proportion (30–40%) of β-structure (Lonngren *et al.,* 1976).

Three experimental findings suggested that the GS I lectin was not a single homogeneous molecular species: 5 bands by alkaline PAGE, less than stoichiometric amounts (*ca* 0.5) of methionine/subunit, and non-sigmoidal inhibition curves generated, when N-acetyl-D-galactosamine and its derivatives were assayed for their ability to inhibit the GS I — guaran precipitation reaction (Hayes and Goldstein, 1974; Murphy and Goldstein, 1977, 1979).

Indeed, GSI. plant seed isolectins comprise a family of tetrameric α-D-galactopyranosyl-binding glycoproteins, composed of various combinations of two different kinds of subunits designated A and B. Subtypes of the A and B subunits have been demonstrated by isoelectric focusing (Murphy and Goldstein, 1979).

Advantage is taken of the different carbohydrate binding specificity of the two types of subunits in order to resolve the mixture. The A subunit exhibits a primary specificity for N-acetyl-α-D-galactosaminyl groups, but also cross-reacts with α-D-galactosyl groups, whereas the β-subunit shows a rather sharp specificity for α-D-galactosyl units. The isolectins, designated A_4, A_3B, A_2B_2, AB_3 and B_4, all bind to a melibionate-Bio Gel column. The A_4 and A_3 B isolectins were displaced from the melibionate-column as purified components by increasing quantities of N-acetyl-D-galactosamine (Murphy and Goldstein, 1977). The A_2B_2, AB_3 and B_4 components after elution from the melibionate column with methyl α-D-galactoside, were resolved on a column of insolubilized blood group A substance. The latter affinity column has N-acetyl-α-D-galactosaminyl determinant groups which interact with the binding sites of the A subunit. A more recent procedure allows separation of all the 5 isolectins on a single affinity column which takes advantage of the differential interaction of the A and B subunits with a *p*-minophenyl β-D-galactopyranoside-derivatized column matrix (Delmotte and Goldstein, 1980).

Physico-chemical characterization of the A_4 and B_4 isolectins reveals the A and B subunits to be closely related glycoproteins. The carbohydrate composition of the two subunits is identical; determined to the nearest integer, both contain N-acetyl glucosamine (2 mol), mannose (8 mol), xylose (2 mol), fucose (2 mol) and traces of arabinose, glucose and galactose (Murphy and Goldstein, 1979). The amino acid composition of the A and B subunits (table 1) are very similar suggest-

Table 1. Amino acid composition of *G. simplicifolia* A_4 and B_4 isolectins[a]

	A_4	B_4
Lys	12	9
His	2	2
Ammonia	29	35
Arg	7	7
Asp	36	33
Thr	20	21
Ser	30	31
Glu	14	19
Pro	12	13
Gly	16	15
Ala	19	19
1/2-Cys[b]	1	1
Val	18	19
Met	0	1
Ile	13	11
Leu	21	19
Tyr	10	9
Phe	16	16
Trp[c]	8	7

[a] Data are expressed in terms of amino acid residues (nearest integer) per subunit.

[b] Determined by hydrolysis in the presence of dimethyl sulphoxide.

[c] Determined spectrophotometrically.

ing that they are homologous structures; in fact rabbit antisera to the A_4 isolectin cross reacts extensively with the B_4 isolectin and *vice versa.* There is one notable difference between the two subunits: the B subunit has one methionine residue whereas the A subunit contains no methionine.

A large number of precipitin reactions of *G. simplicifolia* I isolectins A_4 and B_4 with naturally occurring biopolymers and carbohydrate-bovine serum conjugates have been carried out (Murphy and Goldstein, 1977, 1979; Wood *et al.,* 1979). Both A_4 and B_4 formed precipitates with biopolymers containing multiple, nonreducing α-D-Gal*p* end groups; these include guaran (a galactomannan) and type B blood group substance. Biopolymers containing multiple, nonreducing β-D-Gal*p* end groups such as larch arabinogalactan, did not form precipitates with either isolectin (Murphy and Goldstein, 1979).

A_4 precipitated well with blood groups A_1, A_2, B and precursor substances, with A_2 precipitating less strongly than did A_1 substance; H, Le^a and Le^b substances did not react (Wood *et al.,* 1979). Precipitin inhibition and competitive binding assays confirmed the precipitin data that A_4 is most specific for terminal nonreducing α-linked 2-acetamido-2-deoxy-D-galactopyranose, but also reacts with oligosaccharides with terminal non-reducing α-linked-D-galactosyl end groups (Wood *et al.,*

1979). B_4 precipitated well with B blood group substances and with a precursor substance to a lesser extent, but did not react with A_1 A_2, H, Le^a or Le^b substances (Wood *et al.,* 1979).

Interestingly, a *p*-azophenyl α-D-galactopyranoside-bovine serum albumin (BSA) conjugate did not precipitate with either A_4 or B_4, whereas *p*-azophenyl β-D-galactopyranoside-BSA reacted very weakly with A_4 and strongly with B_4. The *p*-azophenyl β-lactoside-BSA conjugate did not form a precipitate with either A_4 or B_4. A number of biopolymers containing multiple, nonreducing α-D-GalNAc*p* end groups were also tested for their ability to form precipitates with A_4 and B_4 Type A blood group substance, a streptococcus type C cell wall polysaccharide, and asialo bovine submaxillary mucin precipitated strongly with A_4, whereas only asialo bovine submaxillary mucin precipitated a small amount of B_4.Native bovine submaxillary mucin which probably contains a smaller number of incomplete carbohydrate chains with exposed terminal, nonreducing α-D-GalNac*p* residues reacted weakly with A_4 but not with B_4. A_4 precipitated twice as much *p*-azophenyl-N-acetyl-α- D-galactosamine-BSA as the β anomer (*p*-azophenyl-N-acetyl-β-D-galactosamine-BSA) whereas B_4 did not precipitate either of these conjugates.

The best inhibitors of the *B. simplicifolia* I isolectin B_4-guaran system were Me-α-D-Gal*p,* *p*-nitrophenyl (*p*-NO_2 Ph)-α-D-Gal*p*,and *p*-NO_2 Ph-β-D-Gal*p*.Comparison of Me-α-D-Gal*p* with Me-β-D-Gal*p* and a series of oligosaccharides containing nonreducing α-and β-D-galactopyranosyl end units, reveals that the α-anomer is bound more strongly than the corresponding β-anomer. Comparison of Me-α-D-Gal*p*, Me-β-D-Gal*p*, and D-galactose supports the conclusion that a methoxyl group in the α position contributes positively to stabilization of the B_4 sugar complex, whereas a β-methoxyl group represents a destabilising factor. Substitution of the oxygen atom at the C-1 position of methyl β-D-galactoside by a sulphur atom (methyl β-D-thiogalactoside) produced a 3.5-fold decrease in the inhibiting potency of this glycoside (Murphy and Goldstein, 1979)

The interaction of the *p*-nitrophenyl α- and β-D-galactosides with B_4 is remarkable. In sharp contrast to the results obtained with the α- and β-galactosides with aliphatic aglycons, *p*-nitrophenyl α- and β-D-galactoside were equally inhibitory. In as much as methyl and *p*-nitrophenyl α-D-galactoside were equally potent as inhibitors, it is apparent that the aromatic aglycone does not enhance the capacity of the gatactoside to bind to the lectin. However, *p*-NO_2Ph-β-D-Gal*p* was 6 times more potent an inhibitor than Me-β-D-Gal*p*, indicating a strong enhancement of binding by the aromatic aglycon of this β-D-galactoside. This finding formed the basis of a new procedure for the separation of the GS I isolectins (Delmotte and Goldstein, 1980).

The *B. simplicifolia* I A_4 isolectin also has a preference for the α-anomeric configuration: α-GalNacl*p* groups are bound more strongly than the anomeric β-glycosides. It was also observed that the *p*-nitrophenyl α-and β-D-glycosides of D-GalNAc are 2–4 times better inhibitors than the corresponding methyl glycosides of the same anomeric configuration. These results indicate a role of aromatic aglycons in the binding mechanism of glycosides to the A_4 isolectin.

The disaccharides 3-*O*-(α-D-GalNAc*p*)-D-Gal, 6-*O*-(α-D-GalNac*p*)-D-Gal, and 3-*O*-(α-D-GalNAc*p*)-D-Glc were all excellent inhibitors in the A_4–guaran system, being 7–25% better inhibitors than methyl α-D-GalNAc*p*.

The 2-acetamido-2-deoxy group appears to represent an important binding locus for the A_4-D-GalNAc*p* interaction. The observation that D-GalNAc is 13 times more inhibitory than D-Gal, and 72 times better than 2-deoxy D-galactose supports this view. Furthermore, Me-β-D-GalNAc*p* is 55 times more potent an inhibitor than methyl 2-amino-2-deoxy-α-D-galactopyranoside.

Using deoxy and fluoro galactose derivatives it has been possible to determine whether the *H* or *O* atom of the hydroxyl groups are involved in hydrogen bonding to the lectin. The fact that neither methyl 4-deoxy-4-fluoro-α-D-galactopyranoside (20 mM) nor *p*-nitrophenyl 3-deoxy-3-fluoro-β-D-galactopyranoside (10 mM), inhibited the A_4- or B_4- guaran interaction suggests that the *hydrogen* atom and not the oxygen atoms of the C-3 and C-4 hydroxyl groups are involved in hydrogen bonding to the lectin.

Association constants for the binding of methyl α-D-galactopyranoside (methyl α-D-Gal*p*) and methyl 2-acetarnido-2-deoxy-α-galactopyranoside (methyl α-D-GalNAc*p*)) to three *Griffonia simplicifolia* isolectins (A_4, A_2 B_2, B_4) were determined by equilibrium dialysis and fluorescence enhancement measurements. The A and B subunits appear to have approximately the same K_{assoc} for methyl α-D-Gal*p*: 1.45×10^4, 1.98×10^4, and 2.06×10^4 M^{-1} for A_4, A_2B_2, and B_4, respectively, as determined by equilibrium dialysis (Goldstein *et al.,* 1981). Fluorescence enhancement measurements on B_4 gave an association constant of 2.07×10^4 M^{-1} for methyl α-D-Gal*p* and 1.87×10^3 M^{-1} for methyl β-D-Gal*p*. By equilibrium dialysis, we were able to detect 3.3 (theory, 4.0) methyl α-D-GalNac*p* binding sites for A_4 ($K_{assoc}=1.87\times10^5$ M^{-1}), 1.9 for A_2B_2 ($K_{assoc}=1.19\times10^5$ M^{-1}), and were unable to detect any methyl α-D-GalNac*p* binding sites for B_4 (Goldstein *et al.,* 1981). However, four very weak methyl α-D-GalNac binding sites for B_4 were detected by fluorescence enhancement measurement ($K_{assoc}=1.26\times10^2$ M^{-1}). Thus, the A subunit has an affinity for methyl α-D-GalNac 3 orders of magnitude greater than the B subunit (Goldstein *et al.,* 1981).

Using 4-methylumbelliferyl glycosides, DeBoeck and his colleagues measured binding characterstics of α-D-galactosyl, β-D-galactosyl and 2-acetamido-2-deoxy-β-D-galactosyl groups to A_4 and B_4 (DeBoeck *et al.,* 1981).

Precipitation and hapten inhibition data are in accord with these binding measurements. Toward guaran and type B blood group substance which contain α-D-Gal*p* end groups, all isolectins precipitated the same amount of biopolymer. However, AB_3, A_2B_2, and A_3B, which are mono-, di-, and trivalent for α-D-GalNAc*p*, were differentially precipitated by type A blood group substance which contains α-D-GalNAc*p*-end groups. A_3B precipitated the most, A_2B_2 less, and AB_3, no type A substance (figure 1). These isolectins are being studied in order to evaluate the effect of valence on lectin-cell interaction.

Interestingly, the amount of each individual GS I isolectin varies from seed to seed, each seed displaying an unique distribution of the 5 isolectins (Murphy and Goldstein, 1979; Lamb *et al.,* 1981). In fact, no two seeds are exactly alike in their isolectin content. Some seeds contain only B_4 or A_4; others have only A_4, A_3B and A_2B_2 ; still others contain all the five isolectins (Murphy and Goldstein, 1979; Lamb *et al.,* 1981).

Two possible mechanisms could explain the formation of the five isolectins: the A and B subunits could simply combine randomly following polypeptide transla-

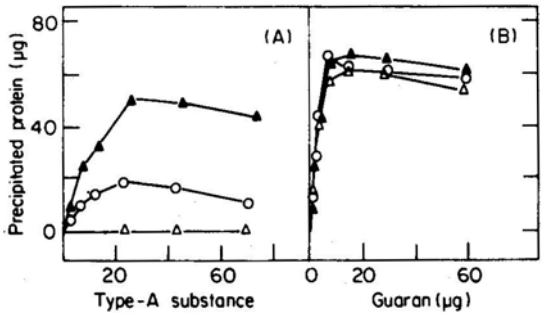

Figure 1. Quantitative precipitation reactions of *B. simplicifolia* I isolectins with glycoconjugates. **A.** Interaction of A_3 B (61 µg) (▲), A_2B_2 (70 Mg) (O), and AB_3 (61 µg) (Δ), with varying amounts of ovarian cyst blood type A substance. **B.** Interaction of A_3 B (61 µg) (▲), A_2B_2 (70 µg) (O), and AB_3 (61 µg) (Δ), with varying amounts of guaran. (Published with permission of *J. Biol. Chem*).

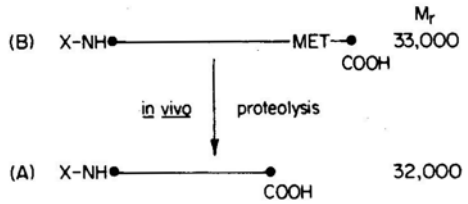

Figure 2. Model showing proposed proteolytic conversion of BS-I B subunit to A subunit. X = pyroglutamate groups.

tion and processing, *e.g.* according to a binomial distribution based on the number of A and B subunits in each isolectin; or a precursor-product relationship may be involved in which proteolytic cleavage of the intact tetrameric B_4 isolectin gives rise sequentially to the remaining four isolectins. A feasible model incorporating these considerations is shown in figure 2.

Alkaline, nondenaturing polyacrylamide gel electrophoresis was employed to analyze the distribution of the five isolectins from 168 single seeds gathered in Ghana from known trees and pods (Lamb *et al.,* 1981). The gels were scanned at 625 nm and the peak height corresponding to each isolectin was measured and expressed as a percentage of the total peak height. Principal components analysis of the correlations among the percentages indicated a sequential relationship among the five isolectins with the concentrations of B_4 and A_4 inversely related Further statistical analyses indicated that although the distribution of isolectins in each seed was unique, seeds gathered from the same tree were more similar than seeds gathered from different trees.

The existence of a precursor–product relationship allows several predictions with regard to the structure of the A and B subunits (figure 2): (i) the N-terminus of both subunits should be identical; (ii) the *C*-terminus of the subunits should differ; and (iii) CNBr-cleavage of the B subunit should yield a small polypeptide and a large piece, identical to the A subunit.

To test these predictions we have carried out chemical studies on the A and B subunits and the preliminary results are presented here.

Carboxypeptidase Y digestion of the A and B subunits gave identical results, the last six *C*-terminal amino adds being: Ser-Thr-Leu-(Phe, Val)-Arg-COOH. Both subunits were blocked at their *N*-terminus by pyroglutamate groups. Removal of these blocking groups with calf-liver pyroglutamate permitted determination of the first 25 *N*-terminal amino acids by automated Edman degradation. There were four differences between the A and B subunits.

Cleavage of the B subunit with CNBr gave a large and a small piece (30,000 and 3,000 daltons, respectively). The large piece was assigned to the *C*-terminus and the small fragment to the *N*-terminus of the B subunit. By SDS-PAGE analysis, the large piece was smaller than the A chain.

Analysis of tryptic peptides by reverse-phase HPLC showed that approximately 30% of the tryptic peptides were common to both the A and B subunits; each subunits also contained unique peptides. The accumulated evidence indicates that the precursor-product relationship is invalid. On the basis of the present studies, it is proposed that the A and B subunits are products of two separate but closely related, possibly contiguous genes.

Labelled with fluorescein, ferritin, colloidal gold and tritium the GSI-B_4 isolectin has been used as a probe for the detection of α-D-galactosyl groups in plant and animal cells. In its immobilized form, the B_4 isolectin was used to isolate the galactomannan from *Cassia alata* seeds (Ross *et al.,* 1976). The B_4 isolectin agglutinates Ehrlich ascites tumour cells and a family of α-D-galactosyl-containing glycoproteins was isolated from an Ehrlich cell membrane fraction (Eckhardt and Goldstein, 1979). Fluoresceinated B_4 was shown to bind to murine kidney cortex sections and we discovered that this α-D-galactosyl binding isolectin could serve as a specific probe for basement membrane of the mouse, rat, rabbit and cow (Peters and Goldstein, 1979).

The GSI-B_4 isolectin has also been shown to be cytotoxic to a variety of animal cell; it was used to generate a variant clone of B_4-resistant 3*T*3 cells (Stanley *et al.,* 1979).

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