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QUARTERLY

The *N*-acetylgalactosamine and lactosamine specific lectin from *Iris hybrida* leaves*

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The lectin isolated from the leaves of *Iris hybrida* binds specifically *N*-acetylgalactosamine and lactose. Its molecule consists of two identical subunits bound by disulfide bonds. The lectin is a glycoprotein containing about 12% of sugars. It binds asialoglycoproteins containing complex type sugar chains. The binding is reduced by half at the concentration of 0.15 to 0.40 mM of the galactose containing disaccharides irrespectively to a type of galactose isomer. This indicates rather broad specificity of *I. hybrida* leaf lectin.

Lectins have been well known for many years to be present in Gramineae (for reviews see [1, 2]). Recently some lectins from other Monocotyledons families, i.e. Aliaceae [3, 4], Amarylidaceae [5–8] and Orchidiaceae [9] were isolated and well characterised. Most of these lectins are specific only towards mannose, differing in this respect from mannose binding dicotyledone lectins which bind glucose as well as mannose [10]. The lectins isolated from all three families of monocotyledones show dimeric or tetrameric structures with the subunit M_r varying from 11500 to 14000 [11]. The only exception is *Allium ascalonicum* agglutinin, a monomeric molecule with M_r of 11000 [3].

In our earlier studies we have found hemagglutinating activity in saline extracts from the rootstocks and leaves of another monocotyledone plant — *Iris hybrida* (Iridaceae [12]). Like in the case of some other monocotyledone lectins [13, 14], we have found some differences in tissue distribution and seasonal changes in the lectin activity of iris extracts. The high agglutinating activity in rootstocks was found during a short period of a few weeks in early spring. In the leaf tissue the activity appears at the time preceding the flowering and increases during the summer months until September [12]. The purpose of this work was to isolate the leaf lectin and compare it with the rootstock lectin.

MATERIALS AND METHODS

Materials. *Iris hybrida* leaves were obtained from Wrocław University Botanical

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Abbreviations: PBS, 10 mM phosphate buffered saline, pH 7.5; TBS, 10 mM Tris-buffered saline, pH 7.5; TBST, Tris-buffered saline containing 0.05% Tween 20; IHA_L, lectin from *Iris hybrida* leaves.

Garden. Human haptoglobin was a kind gift from Dr I. Kątnik, Dept. Chemistry, Wrocław Academy of Medicine. Fetuin and asialofetuin were purchased from Boehringer Mannheim.

Protein was determined by the method of Lowry *et al.* [15] or by the bicinchoninic acid procedure [16] with bovine serum albumin as a standard.

Neutral sugars were determined by the phenol/H_2SO_4 procedure [17] with mannose as a standard.

Hemagglutinating activity was measured on the microtiter U-shaped plates using a 3% suspension of human 0-group erythrocytes in PBS. The inhibitory effect of sugars was measured by incubating two or four hemagglutinating units of the lectin with serially diluted sugars before addition of erythrocytes. The lowest concentration of sugar causing 50% inhibition was determined.

Haptoglobin desialylation was performed by mild acid hydrolysis in 0.025 M sulfuric acid at 60°C for 4 h according to Duk *et al.* [18].

Immobilization of asialofetuin and affinity chromatography. Commercial asialofetuin was immobilised on Sepharose 4B according to manufacturer's instructions. The preparation released from DEAE-Sephadex was applied to a column (1.5 cm × 10 cm) of asialofetuin-Sepharose, equilibrated with 0.1 M Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl and 0.001 M each $MgCl_2$, $MnCl_2$ and $CaCl_2$. The column was washed with starting buffer until $A_{280} < 0.05$, then the proteins bound to the column were released with 0.1 M acetic acid. The flow rate was 15 ml/h.

Polyacrylamide gel electrophoresis was performed according to Laemmli [19] in 12.5% gel. Samples were denatured in the presence of 2% SDS and 2% β-mercaptoethanol by heating for 3 min at 100°C. The proteins were stained with 0.1% Coomassie Brilliant Blue or with silver nitrate according to Merril *et al.* [20]. Phosphorylase *b* from rabbit muscles (M_r 94000), bovine serum albumin (M_r 67000), hen ovalbumin (M_r 43000), human erythrocytes carbonic anhydrase (M_r 30000), soybean trypsin inhibitor (M_r 20100) and bovine α-lactalbumin (M_r 14400) were used for relative molecular mass calculations.

Gas-liquid chromatography. Monosaccharides were determined by gas-liquid chromatography as alditol acetates after hydrolysis of the lectin samples in 2 M trifluoroacetic acid at 105°C for 6 h. Sugar derivatives were separated on the capillary column Hp-5 (0.32 mm × 30 m), using the temperature gradient of 160–230°C (5°C/min for pentoses and 2°C/min for hexoses and hexosamines). *N*-methylglucamine was used as internal standard.

Biotinylation of lectin. The purified lectin was biotinylated with biotinamidocaproate-*N*-hydroxysuccinimide ester (Sigma) according to Duk *et al.* [18]. The biotin ester (500 µg) was dissolved in 50 µl methanol and diluted to 2 ml with PBS. The lectin sample was mixed with the biotin ester solution in the ratio of 1:2 (w/w). The mixture after dialysis against TBS was adjusted to the final concentration of 0.02% lectin and preserved with 0.1% NaN_3.

Dot-blots. Fetuin, asialofetuin, haptoglobin or asialohaptoglobin (0.5 µg each) were spotted on nitrocellulose membranes which were blocked with 0.5% gelatine in TBS, incubated with biotinylated IHA_L (1 µg/ml TBS) for 1 h and then with extr/Avidin-alkaline phosphatase-conjugate (Sigma, 1:20000). The membranes were stained to visualize the enzyme activity with 0.02% 5-bromo-4-chloro-3-indolyl-phosphate and 0.05% 4-nitroblue tetrazolium chloride (Boehringer Mannheim) in 0.1 M Tris/HCl, pH 9.5, containing 0.05 M $MgCl_2$.

The microtiter plate lectin binding assay. The plates were coated with serially diluted fetuin, asialofetuin, haptoglobin or asialohaptoglobin overnight at 4°C. The plates were washed 5× with TBST, incubated with biotinylated lectin (0.5 µg/well) for 2 h at 37°C, then washed 5× with TBST and incubated for 1 h with extr/Avidin-alkaline phosphatase-conjugate (1:10000). The activity was localized using *p*-nitrophenyl phosphate (1 mg/ml) in 0.05 M carbonate buffer, pH 9.6, containing 1 mM $MgCl_2$. The absorbance at 405 nm was read after 1 h incubation. For the inhibition tests, 0.2 µg of biotinylated IHA_L was incubated for 0.5 h with

serially diluted samples of the following sugars: Galβ(1→6)GlcNAc, Galβ(1→3)GlcNAc, Galβ(1→3)GalNAc, Galβ(1→4)GlcNAc, Galα(1→6)Glc. Then the samples were transferred into plate wells coated earlier with 0.5 μg of asialofetuin and incubated for 2 h at 37°C. Next the plates were washed with TBST and incubated with extr/Avidin-alkaline phosphatase. The phosphatase reaction product was detected as described above.

RESULTS AND DISCUSSION

Lectin specificity and isolation procedure

Dialysis of saline extracts from iris leaves against 0.1 M phosphate buffer, pH 7.5 caused a significant loss of hemagglutinating activity. Ion exchange chromatography on DEAE-Sephadex A-50 allowed to divide agglutinating compounds of the extract into two fractions. In the non-binding fraction the agglutination was due to low M_r compounds, which are able to penetrate through the dialysing tube. The chemical character of these compounds so far has not been examined. Conrad & Rüdiger [21] suggested that some agglutinating activity in the fungus *Pleurotus ostreatus* tissues was caused by tannins. Lack of precipitates with coffeine in our preparations excludes the possibility of the presence of tannins in the iris extracts. It seems possible, that iris leaves contain a low relative molecular mass agglutinin (M_r < 5000), as the existence of such agglutinins in plant tissues was already suggested by the group of Ahmed [22, 23]. The low M_r agglutinating compound was responsible for about 80% of total agglutinating activity in iris leaves. Isolation and characterization of this agglutinin needs further studies.

Another agglutinating fraction bound to DEAE-Sephadex in 0.1 M phosphate buffer, pH 7.5, and released from the ion exchanger with 0.5 M NaCl in the same buffer corresponds to the remaining 20% of agglutinating activity in the leaf tissue. Our efforts were aimed at isolation of this protein, as we supposed that it could be similar or the same as the rootstock agglutinin.

Sugar specificity of the leaf lectin partially purified by ion-exchange chromatography was analysed in hemagglutination tests and the results compared with the specificity of rootstock lectin (Table 1). GalNAc, lactose and galactose were found to be the most effective inhibitors of both lectins. The leaf lectin was inhibited also by melibiose. The inhibitory effect of mannose, GlcNAc, arabinose, xylose and glucose was not observed or observed only at a high sugar concentration. Both rootstock and leaf lectins thus appear to be GalNAc/Lac specific. This feature distinguishes *Iris* sp. from other monocotyledons, as only mannose-specific (Orchidiaceae, Aliaceae, Amarylidaceae [6, 7, 24]) or GlcNAc-specific lectins (Gramineae [1, 2]) were found in these plants so far. The only exception reported so far was the Gal/GalNAc lectin from *Colchicum autumnale* [25], probably similar to iris agglutinin. On the other hand, GalNAc/Lac specific lectins are commonly present in dicotyledons [10].

Partially purified IHA_L was subjected to affinity chromatography on asialofetuin-Sepharose 4B. All the activity present in the preparation was bound to the column. The

Table 1. Carbohydrate binding specificity of iris lectins*

Saccharide	Concentration for 50% inhibition of agglutinating activity (mM)	
	Leaves	Rootstocks [12]
Glucose	**	200
Mannose	200	**
Galactose	5.0	6.25
GlcNAc	200	100
GalNAc	2.5	1.25
Arabinose	100	100
Xylose	**	**
Fucose	**	200
Lactose	2.5	3.15
Melibiose	25	not examined

*The concentration necessary for 50% inhibition of human 0-group erythrocytes agglutination with 4 hemagglutinating units;

**no inhibitory effect at 200 mM concentration of sugar was observed.

lectin was eluted with 0.1 M acetic acid, which was more efficient than elution with lactose. The lectin fraction was neutralized, dialysed against PBS and concentrated.

Electrophoretic analysis

Affinity chromatography yielded an electrophoretically homogeneous preparation, both when stained with Coomassie Brilliant Blue or with silver nitrate (Fig. 1). If the denaturation procedure with β-mercapthoethanol at 100°C was omitted we obtained also a single band but of much lower electrophoretic mobility. The M_rs calculated with appropriate standard proteins were 32000 and 64000, respectively. Thus the native lectin appears to be a dimer composed of two identical subunits bound by disulfide bonds. The same results were obtained upon analysis of the rootstock lectin; therefore we suggest that the lectin isolated from the leaf tissue is the same as that present in rootstocks.

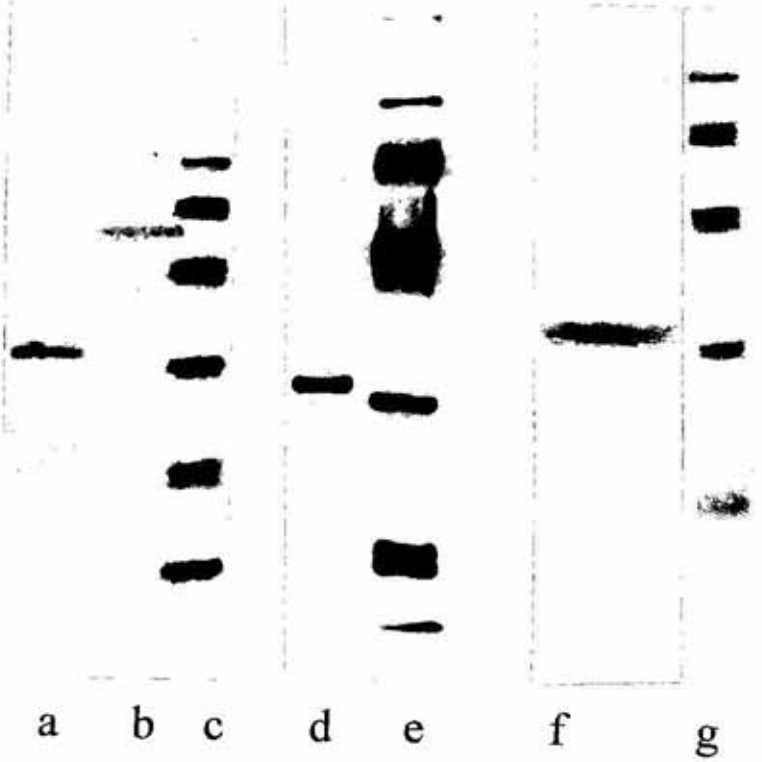

Figure 1. SDS-polyacrylamide gel electrophoresis of the *I. hybrida* lectins.

Lanes (a) and (d), IHA_L after thermal denaturation with 2% β-mercaptoethanol; lane (b), IHA_L, the denaturation procedure was omitted; lane (f), lectin from iris rootstock after denaturation; lanes (c), (e) and (g), M_r reference proteins: phosphorylase *b* from rabbit muscle (94000), bovine serum albumin (67000), hen ovalbumin (43000), carbonic anhydrase from bovine erythrocytes (30000), soybean trypsin inhibitor (20100) and bovine α-lactalbumin (14400). Lanes (a)–(c) and (f)–(g) were stained with Coomassie Brilliant Blue, lanes (d)–(e) with silver nitrate.

Sugar composition

IHA_L is a glycoprotein containing about 12% of sugar. Its sugar composition determined by gas-liquid chromatography (Table 2) suggests the presence of at least one N-glycan chain per each protein subunit. Further investigations are necessary to determine chain types and the manner of incorporation of xylose and arabinose.

IHA_L interaction with glycoproteins and specificity of the lectin towards disaccharides containing galactose

The interaction of biotinylated IHA_L with fetuin, asialofetuin, haptoglobin and asialohaptoglobin was examined in dot-blots and in microtiter plate binding assays (Fig. 2). In both experiments binding was observed only in the case of asialoglycoproteins. This indicates that the presence of terminal sialic acids in sugar chains inhibits the interaction of iris lectin with glycoproteins. The decrease of reactivity towards sialylated glycoconjugates was earlier reported for some dicotyledone lectins [26, 27].

We have found only slight differences in the lectin binding properties of oligosaccharides containing galactose. Fifty percent inhibition of lectin binding was obtained at the concentration range of 0.15–0.40 mM of four disaccharides (Fig. 3), irrespectively of the type of monosaccharide bound to galactose and the position of binding. The broad specificity of iris lectin indicates that it differs from other

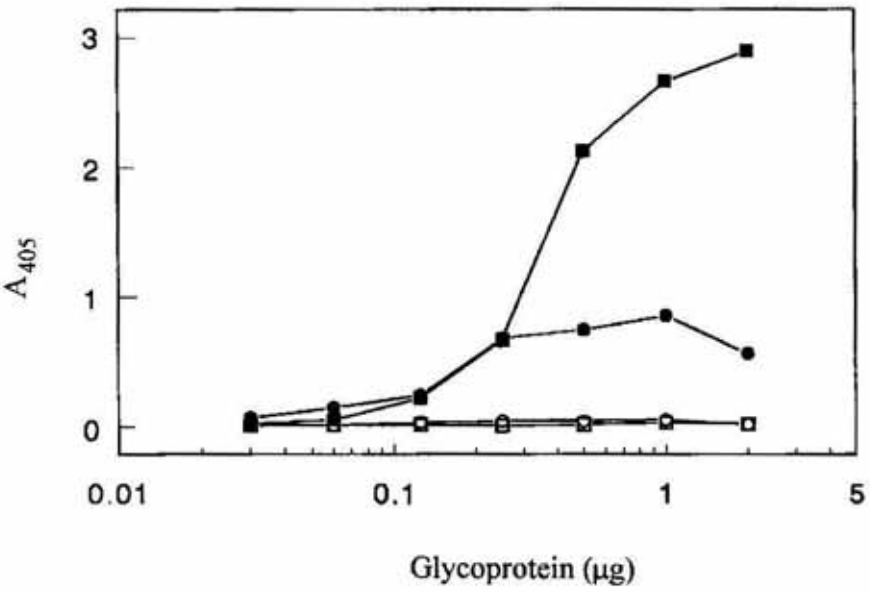

Figure 2. Binding of *I. hybrida* lectin to microtiter plates coated with glycoproteins.

The plates were coated with serially diluted fetuin (○), asialofetuin (●), haptoglobin (□) or asialohaptoglobin (■), starting from 2 μg/well. After being washed the plates were incubated with biotinylated lectin at the constant concentration of 0.2 μg/well.

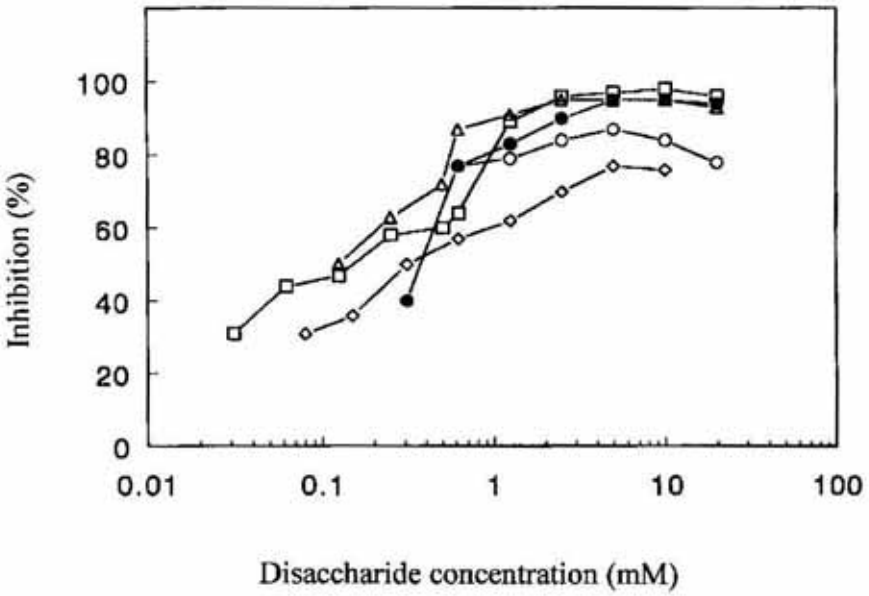

Figure 3. The inhibitory effect of disaccharides on binding of *I. hybrida* lectin to asialofetuin.

(○) Galβ(1→6)GlcNAc, (●) Galβ(1→3)GlcNAc, (△) Galβ(1→3)-GalNAc, (□) Galβ(1→4)GlcNAc, (◇) Galα(1→6)Glc. The lectin samples (0.5 μg/well) were incubated with serially diluted sugars (starting from 20 mM concentration) and then transferred to a plate coated with asialofetuin (0.5 μg/well). After incubation the plate was washed and incubated with extr/Avidin-alkaline phosphatase (Sigma, 1:20000). The enzyme activity was estimated in carbonate buffer, pH 9.5, containing $MgCl_2$.

Table 2. Sugar composition of the lectin from *Iris hybrida* leaves.

Carbohydrate composition is given as mole residue per mole subunit of M_r 32000

Monosaccharide	Mole/subunit
GlcNAc	5
Mannose	17
Galactose	1.75
Arabinose	3.15
Xylose	1.30

lectins such as jacalin, which recognizes only Galβ(1→4)GalNAc [28], or the *Erythrina cristagalli* lectin, specific for lactosamine in N-glycan structures [26].

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