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Note

Inhibitory activity of disaccharide–L-asparagine compounds against hemagglutination by various lectins*

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In view of the importance of lectins (hemagglutinins) as reagents for the isolation and characterization of carbohydrate-containing macromolecules[1], and for the elucidation of the chemical structure of the receptor sites at the surface of animal cells[2–4], it is of interest to determine their carbohydrate binding-specificity with hapten inhibitors of well characterized chemical structure.

Characterization with mono- and oligo-saccharides and their glycosides may lead to erroneous interpretations when applied to the structure elucidation of glycoproteins[5], as the presence of the peptide chain may influence the inhibitory activity of the linked oligosaccharide residues. Therefore, purified plant lectins from *Cytisus sessilifolius*, *Ulex europeus* (gorse or furze Seed), *Lens culinaris* (lentil), *Canavalia ensiformis* (concanavalin A of the jack bean), *Solanum tuberosum* (potato), and *Triticum vulgaris* (wheat germ) were studied by hemagglutination inhibition tests with di-*N*-acetylchitobiose and with derivatives having one residue (or two) of 2-acetamido-2-deoxy-D-glucose linked as *N*-glycosyl to asparagine. In order to test the role of the L-asparagine residue present in the core structure of *N*-glycoproteins, and of modifications of the (1→4) linkage of the di-*N*-acetylchitobiose residue linked to the L-asparagine residue, in the inhibition of hemagglutination by lectins, various synthetic L-asparagine–oligosaccharides available in this laboratory were investigated with lectins known to react with either 2-acetamido-2-deoxy-D-glucose or oligosaccharides containing this residue.

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RESULTS AND DISCUSSION

Although the carbohydrate-binding specificities of the lectins just mentioned have been reported[1], the influence, on the inhibition of hemagglutination, of an amino acid (or peptide) attached to the carbohydrate residue has not been reported, except for the binding specificity of wheat-germ agglutinin[1]. The six lectins were tested with 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine [**1**, β-D-Glc*p*NAc-(1→4)-Asn], 2-acetamido-3- [**2**, β-D-Glc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→4)-Asn], -4- [**3**, β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn], and -6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine [**4**, β-D-Glc*p*NAc-(1→6)-β-D-Glc*p*NAc-(1→4)-Asn], and 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucose [**5**, β-D-Glc*p*NAc-(1→4)-D-GlcNAc, di-*N*-acetylchitobiose]. The results of the inhibition tests were compared to those of isolated compounds reported earlier, and are reported in Table I.

The best inhibition of hemagglutination by the *Cytisus sessilifolius* (CSA) lectin was observed[6,7] with di-*N*-acetylchitobiose (**5**). The inhibition with the β anomers of the *p*-nitrophenyl glycosides of di-*N*-acetylchitobiose [*p*NP-β-D-Glc*p*NAc-(1→4)-D-GlcNAc] and of its (1→6) analog[7] [*p*NP-β-D-Glc*p*NAc-(1→6)-D-GlcNAc] suggests a specificity of this lectin for a β-D-(1→4) linkage. The requirement of a (1→3)- or a (1→4)-glycoside linkage for β-D-linked oligosaccharides in order to bind the CSA was reported[1]. β-D-Glc*p*NAc-(1→4)-Asn (**1**) did not show any inhibitory activity against this lectin, and we observed that β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn (**4**) was more inhibitory than β-D-Glc*p*NAc-(1→4)-D-GlcNAc.

TABLE I

INHIBITION TESTS[a]

Sugar	*CSA*	*UEA*	*STA*	*WGA*	*LCA*	*Con A*
D-GlcNAc	>20[b]	>10[c]	>354[d]	53[e]	5	5
β-D-Glc*p*NAc-(1→4)-L-Asn (**1**)	>10	>10	1.25	>10	2.5	>10
β-D-Glc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→4)-L-Asn (**2**)			>10	>10		
β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-L-Asn (**3**)	0.63	2.5	1.25	0.63	>10	>10
β-D-Glc*p*NAc-(1→6)-β-D-Glc*p*NAc-(1→4)-L-Asn (**4**)	>10	>10	>10	>10	>10	>10
β-D-Glc*p*NAc-(1→4)-D-GlcNAc (**5**)	2.5	5	0.31 0.32[d]	0.31 1.7[e]	>10	>10
(β-D-Glc*p*NAc-1→4)$_2$-D-GlcNAc		1.25[c]	0.25[d]	0.05[e]		
(β-D-Glc*p*NAc-1→4)$_3$-D-GlcNAc		2.5[c]	0.03[d]	0.07[e]		
(β-D-Glc*p*NAc-1→4)$_4$-D-GlcNAc		2.5[c]	0.02[d]	0.04[e]		
*p*NP-β-D-Glc*p*NAc-(1→4)-D-GlcNAc	1.25[b]					
*p*NP-β-D-Glc*p*NAc-(1→6)-D-GlcNAc	>20[b]					

[a]Minimum amounts (mg/mL) completely inhibiting 4 hemagglutinating doses. [b]From Ref. 7. [c]From Ref. 8. [d]Calculated from Ref. 12. [e]Calculated from Ref. 9.

The specificity of the *Cytisus*-type anti-H(O) lectin from *Ulex europeus* seeds (UEA) is very similar to that of *Cytisus sessilifolius*, and inhibition of hemagglutination was reported[8] with di-*N*-acetylchitobiose (**5**). No inhibitory activity was observed with β-D-Glc*p*NAc-(1→4)-Asn (**1**), and β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn (**4**) was found to be a better inhibitor than β-D-Glc*p*NAc-(1→4)-D-GlcNAc. The presence of the asparagine residue linked as aglycon in the β-D configuration increases the inhibitory activity of the parent disaccharide in the case of the two *Cytisus*-type lectins.

The potato (*Solanum tuberosum*) lectin (STA), a blood-group nonspecific hemagglutinin that is specifically inhibited by oligosaccharides containing 2-acetamido-2-deoxy-D-glucose[9], shows an inhibitory activity increasing with increase in chain length of the carbohydrate receptor, up to the tetraose, which suggests an extended binding-site[9]. 2-Acetamido-2-deoxy-D-glucose itself is not an inhibitor[9], nor are several disaccharides containing a 2-acetamido-2-deoxy-D-glucose residue in the reducing position[10]. Thus, it was rather unexpected that the glycosylamino acid **1** would show a strong inhibition of the hemagglutination by STA. The presence of the asparagine residue linked to di-*N*-acetylchitobiose slightly decreased the inhibitory activity with this lectin, but β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-L-Asn (**3**) still remained a good inhibitor, whereas the (1→3) and the (1→6) isomers (**2** and **4**) were inactive, a result that is in agreement with the observation[1] that the lectin from potato tubers exhibits a carbohydrate-binding specificity directed towards β-D-(1→4)-linked oligomers of 2-acetamido-2-deoxy-D-glucose.

A dimeric, blood-group nonspecific lectin, namely, wheat-germ agglutinin (WGA), exhibits binding specificity toward 2-acetamido-2-deoxy-D-glucose and its β-D-(1→4)-linked oligomers[3,9,11-13]; its combining site appears to be complementary to a sequence of three β-D-(1→4)-linked 2-acetamido-2-deoxy-D-glucose units[14]. As observed by Levine *et al.*[15], and later studied by Bhavanandan and Katlic[16] and Peters *et al.*[17], nonspecific binding of *N*-acetylneuraminic acid also occurs. Nagata and Burger[18] and Privat *et al.*[13] predicted that each wheat-germ agglutinin dimer has four equivalent binding-sites, each consisting of at least three subsites. The recent X-ray crystallographic studies of Wright[19], which demonstrated the existence of primary and secondary binding-locations, showed that the binding mode of 2-acetamido-2-deoxy-D-glucose oligomers differs markedly from that of *N*-acetylneuraminic acid oligosaccharides. An important observation of Wright[19] was that the acetamido group of both 2-acetamido-2-deoxy-D-glucose and *N*-acetylneuraminic acid interacts with protein side-chains. The data of Allen *et al.*[9] and Goldstein *et al.*[20] suggested that an uncharged amide group (*e.g.*, *N*-acetyl) must be present on C-2 of the pyranose form of the sugar, and that only derivatives of 2-acetamido-2-deoxy-D-glucose having a free hydroxyl group on C-3 are inhibitors of the hemagglutination with wheat-germ agglutinin.

The recent results of Monsigny *et al.*[21] confirmed that the 2-acetamido group and an adjacent hydroxyl group (on C-3 in 2-acetamido-2-deoxy-D-glucose and on C-4 in *N*-acetylneuraminic acid, both equatorially attached) are the main determinants

in the binding. The lack of inhibition of wheat-germ agglutinin hemagglutination by the (1→3) (**2**) and the (1→6) isomer (**4**) of di-*N*-acetylchitobiose is in accord with these findings, and confirms the suggestion of Uhlenbruck *et al.*[22], Privat *et al.*[13], and Shier[23] that the wheat-germ agglutinin receptor-site has the partial structure of β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn (**4**). Compound **1** did not show any inhibitory activity against this lectin, confirming previous observations[9,20]. The results of recent studies[24] suggested that the presence of an intact, reducing-terminal 2-acetamido-2-deoxy-D-glucose residue was absolutely necessary for the binding to wheat-germ agglutinin. However, the presence of an L-asparagine residue bound to that sugar residue does not strongly influence the binding, as **3** was found to be a good inhibitor, its activity being ~50% of that of **5**; as expected, **2** was inactive.

Hemagglutination by the lentil lectin (*Lens culinaris*, LCA) is inhibited[25] by the so-called Mäkelä's group-III sugars[26], D-mannose being the most active; 2-acetamido-2-deoxy-D-glucose is a good inhibitor, slightly less active than D-glucose. The presence of free hydroxyl groups on C-3, -4, or -6, as well as the configuration of OH-3 and -4, was shown[27] to be critical for the binding to the lectin, whereas the substitution at C-2 was less important. Also, 3-*O*-substituted derivatives of D-glucose were shown to be better inhibitors than D-glucose itself[28]. In view of the suggestion that amino groups are not involved in the carbohydrate-binding site of the lectin[29], it was rather unexpected that the glycosylamino acid **1** would show a strong inhibition of the hemagglutination by LCA. This blood-group nonspecific, mitogenic lectin can interact either with terminal residues or with internal core saccharides[3], a property shared by a few other lectins, *e.g.*, concanavalin A.

The carbohydrate-binding specificity of LCA is rather complicated, and simple sugars that are good hapten-inhibitors, such as D-mannose or 2-acetamido-2-deoxy-D-glucose, do not strongly inhibit the hemagglutination with this lectin. Kornfeld *et al.*[30] observed that the red-cell glycopeptide was a potent inhibitor of LCA, and that the glycopeptide of IgG immunoglobulin [having an internal β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn structure] was an even stronger hapten-inhibitor. Studies by the Kornfelds[2] established that the inhibitory activity of this lectin depends not only on the sequence of the sugar residues of the receptor, but also on the location of the glycosidic linkages, the β-D-(1→4) linkage showing strong activity, whereas the β-D-(1→6) linkage is devoid of activity. The observation that the (1→6) isomer (**4**) of **3** did not inhibit the hemagglutination with LCA is in agreement with the results of previous studies[2].

The carbohydrate-binding specificity of concanavalin A (con A) has been studied extensively[1,31,32]; it is similar to that of LCA (ref. 33), but con A seems[25] to bind a longer part of the sugar chains located on cell surfaces than does LCA. Its carbohydrate-binding site has been shown to be complementary to three or more sugar units[1]. Concanavalin A also binds to isomers or derivatives at C-2 of D-glucose, and 2-acetamido-2-deoxy-D-glucose is 50% as active as D-glucose itself[1]; the presence of the uncharged acetamido group is necessary for the binding[34]. However, this lectin exhibits pronounced anomeric specificity for the α anomers of D-manno- and

D-gluco-pyranose oligosaccharides[20,34]; it has been suggested[35] that O-5 of the α-D anomer contributes to the binding energy of the con A–carbohydrate complex.

The results of studies[36] with immunoglobulin glycopeptides suggested that terminal 2-acetamido-2-deoxy-β-D-glucopyranosyl residues may interact in a non-specific way with con A; on the basis of the inhibition studies of Smith and Goldstein[34], however, an interaction with the protein moiety is possible. The presence of a hydrophobic region adjacent to the specific, carbohydrate binding-site that interacts with aromatic β-D-glucopyranosides was proposed[1], which emphasizes the influence of the aglycon on the inhibitory activity. In the case of sophorose (2-*O*-β-D-glucopyranosyl-D-glucose), which is a good inhibitor of con A hemagglutination[37], the three hydroxyl groups of the reducing D-glucose residue were implicated in the binding, as β-D-glucopyranosyl residues of oligo- and poly-saccharides are not inhibitory[20,34]. This, and the results of nonspecific interaction between con A and heparin[38], as well as complex-formation with various glycoproteins[1], prompted us to include this lectin in our studies. None of the compounds tested, however, showed any inhibitory activity in the con A hemagglutination.

The results of this study demonstrate that the inhibitory activity of carbohydrate residues depends not only on the configuration of the nonreducing, carbohydrate end-group, but also on the configuration of its interglycosidic linkage and the presence of an amino acid (asparaginyl) residue linked to the carbohydrate residues.

EXPERIMENTAL

Preparation of lectins. — *Cytisus sessilifolius* (CSA)[39], *Ulex europeus* (*Cytisus*-type, UEA)[8], *Solanum tuberosum*[40] (STA), and wheat-germ agglutinin (WGA)[18] were respectively prepared according to Matsumoto and Osawa[8,39], Marinkovich[40], and Nagata and Burger[18], *Lens culinaris* syn. *esculenta* agglutinin (LCA), as previously described[22], and concanavalin A, by affinity chromatography[41]. All the lectins were homogeneous by ultracentrifugal analysis and poly(acrylamide) disc-gel electrophoresis.

Preparation of inhibitors. — The following compounds were prepared according to procedures reported earlier: 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine[42] ("*N*-acetyl-D-glucosamine-L-asparagine") [β-D-Glc*p*NAc-(1→4)-Asn] (**1**), 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine[43] ("di-*N*-acetylchitobiose-L-asparagine") [β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn] (**3**), 2-acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine[44] ("di-*N*-acetylisochitobiose-L-asparagine") [β-D-Glc*p*NAc-(1→6)-β-D-Glc*p*NAc-(1→4)-Asn] (**4**), and di-*N*-acetylchitobiose[45] [β-D-Glc*p*NAc-(1→4)-D-GlcNAc] (**5**).

*2-Acetamido-3-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine* [β-D-*Glc*p*NAc*-(1→3)-β-D-*Glc*p*NAc*-(1→4)-*Asn*] (**2**). — A solution of 2-acetamido-3-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-

β-D-glucopyranosyl)-4,6-di-*O*-acetyl-*N*-[benzyl *N*-(benzyloxycarbonyl)-L-aspart-4-oyl]-2-deoxy-β-D-glucopyranosylamine[46] (0.2 μmol) in methanol (0.2 mL) was treated overnight with a methanolic solution of sodium methoxide (0.02 μM) at room temperature. The solvent was evaporated *in vacuo*, and the residue [one major spot in t.l.c., having R_F 0.3 on cellulose in 2:4:1 (v/v) butanol–acetic acid–water] was used without further purification.

Inhibition tests. — The tests were performed as previously described[7]. Human O erythrocytes, freshly obtained from a donor, were washed three times, and used as a 3% suspension.

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